Oxidative stress related genes in cassava post-harvest physiological deterioration

Reilly, Kim

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Oxidative Stress Related Genes in Cassava Post-harvest Physiological Deterioration

Submitted by
Kim Reilly

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Signature

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

For Pat

"Brothers and Sisters:

Many words walk in the world. Many worlds are made. Many worlds are made for us. There are words and worlds which are lies and injustices. There are words and worlds which are truths and truthful. We make true words. We have been made from true words. In the world of the powerful, there is no space for anyone but themselves and their servants. In the world we want everyone fits. In the world we want many worlds to fit. The nation which we construct is one where all communities and languages fit, where all steps may walk, where all may have laughter, where all may live the dawn."

From the Fourth Declaration of the Lacondon Jungle.
Mexico, 1996.

Abstract

Storage roots of cassava (*Manihot esculenta* CRANTZ) undergo a rapid post-harvest physiological deterioration (PPD). The process is an endogenous root disorder which can occur within 24–48 hours after harvest. It is initially observed as blue/black vascular streaking that develops from wound sites and along xylem strands, followed by browning of the storage parenchyma. Several lines of evidence suggest that PPD is an enzymatically mediated oxidative process. Therefore, molecular and biochemical approaches were used to study the generation of reactive oxygen species (ROS) in the cassava storage root after harvest, and to examine the expression of the primary ROS-scavenging enzymes — catalase, peroxidase and superoxide dismutase.

A cassava catalase, MecCAT1, was isolated from a root PPD-related cDNA library constructed 48 hours after harvest. The clone represents a full-length transcript of 1792 bp. It encodes a predicted protein of 492 amino acids with highest similarity to *Ricinus communis* CAT2 protein (91% pairwise amino acid identity). Southern hybridisation indicated the presence of at least two, probably three catalase genes in the cassava genome. The MecCAT1 transcript is expressed predominantly in roots, with low-level expression in leaves, and contains a conserved carboxy-terminal peroxisomal targeting signal, suggesting the protein may be targeted to glyoxysomes within the root. The transcript was up-regulated in response to pre-harvest pruning and ethylene treatment. Catalase protein activity and MecCAT1 transcript expression during the post-harvest period were compared in a range of cultivars showing differing susceptibility to PPD. These data suggest that high levels of catalase activity may play a role in delaying the deterioration process.

An oligo-nucleotide primer based on the conserved active site of plant peroxidases was used to allow screening of the cDNA library by a PCR-based approach. Five positive clones were obtained and two, designated MecPX1 and MecPX2, were further characterized. Both clones were truncated at the 5' and 3' ends and were identical except that MecPX2 was the larger transcript, having additional sequence at both the 3' and 5' ends. The MecPX2 transcript was 726 bp in size, and encoded a predicted protein of 241 amino acids with greatest similarity to a cationic wound and ethylene induced peroxidase, VIRPRX, of *Vincula angularis* (61% pairwise amino acid identity). Southern blotting indicated the cognate gene may be present in the cassava genome in two copies, and forms part of a related gene family comprising at least three members. Northern blotting indicated that the transcript was expressed primarily in roots, with no expression detected in leaf or petiole. The transcript was strongly up-regulated in response to ethylene treatment, but was unaffected by pre-harvest pruning or methyl jasmonate treatment. MecPX2 transcript accumulation and overall peroxidase enzyme activity were compared in a range of cultivars showing differing susceptibility to PPD. This indicated that higher levels of peroxidase expression are correlated with susceptibility to PPD. Polyacrylamide gel electrophoresis indicated at least six peroxidase isoforms in non-deteriorated root, with seven detected in deteriorated root. Localisation experiments using tissue printing and histochemical approaches indicated that peroxidase activity is initially localized to the xylem parenchyma and cortex, and spreads throughout the root parenchyma as the PPD response progresses.

Polyacrylamide gel electrophoresis indicated the occurrence of 4 superoxide dismutase isoforms in the cassava storage root. No change in root SOD isoform pattern was detected over a 5 day time-course. A probe corresponding to a cassava root cytosolic CuZnSOD was generated using a PCR approach. Southern blotting indicated the cognate gene is part of a small gene family of cassava CuZnSODs. Northern blotting indicated the transcript was expressed in storage roots, leaves and petioles of the cassava plant. The transcript was slightly up-regulated by pre-harvest pruning, but unaffected by ethylene or methyl jasmonate. Transcript expression during the post-harvest period was compared in a range of different cultivars showing differing susceptibility to PPD, and indicated no significant differences between cultivars.

Superoxide (O_2^-) was produced within 15 minutes of injury and declined to low levels 6–10 hours post-injury, while hydrogen peroxide (H_2O_2) was produced within 3 hours of injury, peaking within 24–27 hours before declining. These data indicate a transient, wound-induced oxidative burst in cassava storage roots. O_2^- production occurred throughout the root parenchyma, with high levels detected in the cambium. H_2O_2 accumulated initially in the cortex, cambium and region of storage parenchyma directly beneath the cambium, and spread to internal tissue over time. Thin-layer chromatography revealed five secondary compounds with potential *in vivo* free radical-scavenging properties. Three could be identified at a high confidence level as the coumarin scopoletin and the flavonoids gallocatechin and rutin. A fourth was tentatively identified as the flavonoid epicatechin gallate. This suggests a range of easily oxidizable compounds exist in cassava root that could act as reducing agents in either enzymatic or non-enzymatic reactions during the post-harvest period.

During the course of the project, a number of non-preconceived target clones were obtained, including an RNA polymerase subunit (MecRPB8), a translation initiation factor eIF-5a (MecTIF), an aspartic protease (MecASP1), a serine protease (MecSER1) and a cysteine protease inhibitor (MecCPI1). Since a component of the project was the generation of cDNA clones for inclusion on the cassava genetic map, these were subcloned, sequenced and characterized. As proteases and protease inhibitors have been implicated in defence and senescence responses in other plant systems, initial northern blotting experiments were carried out. These indicated that MecASP1 and MecCPI1 are up-regulated in the cassava storage root within 24 and 48 hours after harvest, respectively.

Acknowledgements

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Abbreviations

aa	amino acid
ABA	abscisic acid
AFLP	amplified fragment length polymorphism
Amp	ampicillin
Amp^R	ampicillin resistant
AOS	active oxygen species
bp	base pair
BSA	bovine serum albumin
CAT	catalase
CGIAR	Consultative Groups on International Agricultural Research
CIAT	Cento Internacional de Agricultura Tropical
DAB	3,3 diaminobenzidine tetrahydrochloride
DNase	deoxyribonuclease
DEPC	diethyl pyrocarbonate
DFID	Department for International Developement
DHBS	3,5-dichloro-2-hydroxy benzene sulphonic acid
DMPC	dimethyl pyrocarbonate
DMSO	dimethyl sulphoxide
DPPH	1,1 diphenyl-2-picryl-hydrazl
DTT	dithiothreitol) Sigma. D-8161.
EDTA	ethylenediamine tetra-acetic acid
FW	fresh weight
HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
HR	hypersensitive response
HRGP	hydroxyproline rich glycoprotein
IPTG	isopropylthiogalactoside
IEF	isoelectric focussing
IEF PAG	isoelectric focussing polyacrylamide gel
JA	jasmonic acid
MOPS	3-(N-Morpholino) propanesulphonic acid
NBT	nitroblue tetrazolium.
NO	nitric oxide
PAG	polyacrylamide gel

PAGE	polyacrylamide gel electrophoresis
PAL	phenylalanine ammonia lyase
PEG	polyethylene glycol
PMS	phenazine methosulphate
PPD	post-harvest physiological deterioration
PR	pathogenesis related
PVP	polyvinyl pyrrolidone
PX	peroxidase
R_f	retention factor
RNase	ribonuclease
ROS	reactive oxygen species
ROI	reactive oxygen intermediates
SA	salicylic acid
SAR	systemic aquired resistance
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
Tris	tris(hydroxymethyl) methylamine.
TLC	thin layer chromatography
UTR	un-translated region
UV	ultra violet
X-Gal	5-chloro-4-bromo-3-indoyl-β-d-galactoside

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CHAPTER ONE:
INTRODUCTION

1.1 The cassava plant

1.1.2 Taxonomy, origin and distribution

Cassava (*Manihot esculenta* subsp. *esculenta*), also known as manioc, yuca and tapioca, is a perennial shrub belonging to the Euphorbiaciae, a group which also includes the spurges and agronomically important plants such as *Ricinus communis* (castor bean) and *Hevea brazilienesis* (rubber). A characteristic of the Euphorbiaceae is the presence of lactifers and the production of latex. Although *M.glaziovii* is used as a minor source of rubber, *M.esculenta* is the only widely cultivated member of the 98 described species in the genus *Manihot,* and is primarily cultivated for its edible starchy roots. A number of related species *M.cathaginensis, M.aesculifolia* and *M.leptophylla* have also been reported to produce edible roots, and may have been cultivated historically on a small scale (Allem *et al.* 2000).

Cassava is native to South America with 2 possible centres of origin - Brazil / Paraguay and Mexico / Guatemala (Jennings 1976). Recent results indicate the crop was domesticated from populations of *Manihot esculenta* subsp. *flabellifolia* along the southern border of the Amazon basin (Olson and Schaal 1999). Cassava is one of the earliest cultivated crops and no wild populations are thought to exist today – the population comprises only cultivated clones and landraces (Puonti-Kerlas 1998). After the arrival of the Spanish and Portuguese in South America, it was introduced to Africa and Asia in the 16^{th} and 17^{th} centuries respectively. Today it is cultivated in tropical and subtropical Africa, America, and Asia between 30 ° N and 30 ° S in regions where mean average temperatures exceed 20 ° C and rainfall varies from 500mm to 8000mm (Puonti-Kaerlas 1998).

1.1.2 Morphology of the cassava plant

The cassava plant is a dicotyledenous, diploid (2n = 36) angiosperm cultivated primarily for its starchy tuberous roots, although the lobed palmate leaves may also be eaten. Shrubs produce a woody stem and grow from 1 to 5 metres in height depending on cultivar. Each plant produces 5 - 10 storage roots by secondary root thickening (figure 1.1.1). Cultivars may be distinguished on the basis of a range of morphological characteristics including stem and leaf colour, branching pattern, leaf shape and lobing and root form and colour. Petioles are 5- 30 cm in length, leaves are deeply palmate and the lobe number is a function of cultivar and position on the plant. The number of lamina lobes is usually 5-7, although any number from 3-9 may occur (Onwueme 1978). Not all cultivars produce normal flowers and in some cultivars flowering is rare

or non existent. Cultivars which do produce flowers are monoecious. Female flowers are usually larger than the male, and in each inflorescence the female flowers open about a week prior to the male. Thus the crop is strongly outcrossing and is pollinated primarily by insects (Onwueme 1978). The fruit is a 3-locular capsule 1-5 cm in diameter and matures 3-5 months after pollination, however there is a low seed yield (a maximum of 3 seeds) per pollination (Fregene 1997). Agriculturally, the crop is generally propagated vegetatively via lignified stem cuttings.

1.1.3 The cassava storage root

Each cassava plant gives rise to 5-10 storage roots by secondary thickening. Mature storage roots range in size from 15-100 cm in length and may weigh 0.5-2 kg depending on cultivar and growing conditions. In contrast to other root crops such as yam, potato and sweet potato, the cassava storage root does not exhibit endogenous dormancy and has no propagative function. Adventitious roots of cassava comprise 3 major tissue regions: an outer epidermal layer, a cortex containing parenchymatous cells and a central vascular region containing alternating zones of xylem and phloem in a tetrarch pattern. Initiation of storage root formation begins within 52 to 76 days after planting of the stem cutting (Cabral *et al.* 2000). Storage roots reach maturity and are generally harvested 8 – 18 months after planting depending on cultivar, although they may be left in the ground as a famine reserve for prolonged periods (Cock 1985). The structure of the storage root is shown in figure 1.1.1. An outer periderm and a thin cortex surround a core of mainly starch parenchyma which forms the bulk of the root. Secondary xylem is dispersed throughout the storage parenchyma, secondary phloem is dispersed within the cortical parenchyma (Hunt *et al.* 1977). Two meristematic tissue regions are present – the outer phellogen, which gives rise to the periderm on the outside and the phelloderm or secondary cortex to the inside. The second meristematic region is the cambium, which gives rise to the secondary phloem to the outside and secondary xylem on the inside (Cabral *et al.* 2000).

Root composition has been described as approximately 62-65% water, 32-35% carbohydrate, 0.7-2.6% protein, 0.2-0.5% fat, 0.8-1.3% fibre and 0.3-1.3% mineral matter (Onwueme 1978, Wenham 1995). Most of the carbohydrate fraction is starch which makes up 85% of storage root dry weight (Cock 1985). With regard to mineral content, phosphorus and iron predominate, with low levels of calcium. The roots are relatively rich in vitamin C (35mg per 100g fresh weight) and contain traces of vitamins A, B_1 and B_2, however levels of thiamine and riboflavin are low. Protein content is not

only low in quantity but poor in quality since methionine, lysine, tryptophan, phenylalanine, tyrosine and cysteine are low (Onwueme 1978, Cock 1985).

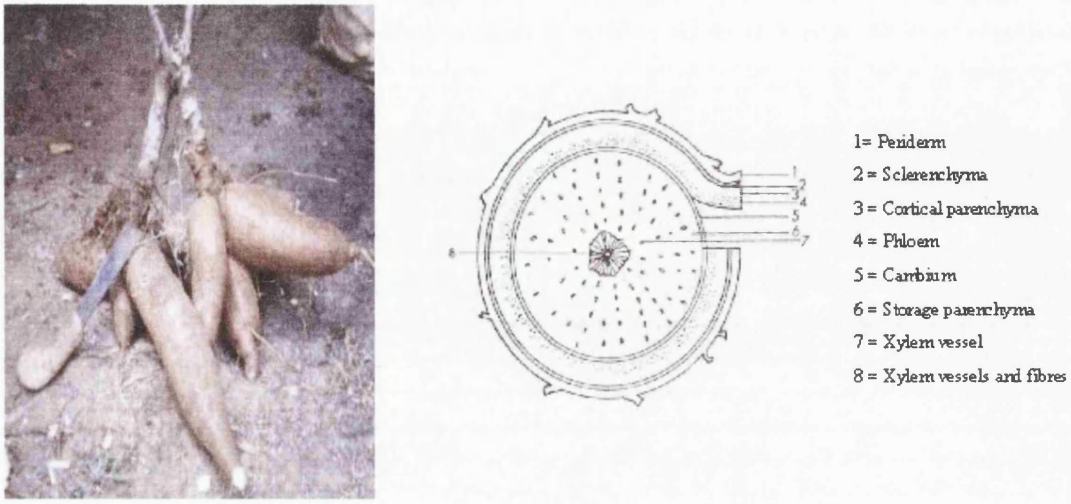


Figure 1.1.1 The storage root of cassava. Freshly harvested storage roots arising from the base of a vertically planted "stake" are shown on the left. A schematic representation of the tissues of the mature cassava storage root (after Hunt *et al.* 1977) is shown on the right. During processing for human consumption parts 1-4 are peeled and the storage parenchyma is processed.

1.2 Agricultural uses and importance of cassava

Traditionally cassava has been grown primarily as a staple food crop particularly by small subsistence farmers in marginal areas. It is a hardy crop with several advantages to the small scale farmer:

- Highly efficient carbohydrate production crop.
- Following an initial establishment period it is tolerant of even prolonged drought
- and other adverse environmental conditions.

  Tolerant of low soil fertility and can produce acceptable yields in nutrient poor soils, or soils containing high levels of aluminium and magnesium where other crop plants such as maize, sorghum, beans and soybeans die soon after germination.
- As there is no fixed period of maturity storage roots can be left in the ground and harvested from 6 months to 3 years after planting, thus effectively acting as a famine reserve.
- Propagation is via lignified stem cuttings or "stakes" thus none of the harvest must be set aside as planting material for the following season.
- Local landraces are well adapted to mixed cropping and subsistence cultivation, allowing farmers to minimise the risk of total crop failure

(Wenham 1995, FAO 1998, Puonti-Kaerlas 1998). It is a staple food crop for over 500 million people living throughout the tropics (FAO 1998), and as a human calorie source cassava has been ranked 4th after rice, sugar cane and maize. In tropical Africa, it has been the single most important source of calories in the diet, with largest production in Nigeria, Democratic Republic of Congo and Uganda. Other uses include as an animal feed and as raw material for the starch industry. Although starch makes up 85% of tuber dry weight, cassava to date accounts for only a small percentage of internationally traded starch (Wenham 1995, Beeching *et al.* 1998). During 1993 - 1995 the distribution of cassava among food, animal feed and industries was 59%, 24% and 17% respectively with the largest projected growth area in the starch industry.

Cassava storage roots do not store well, and must be processed soon after harvest, due to the occurrence of a rapid post-harvest physiological deterioration which forms the basis of this study. In addition, depending on cultivar and growth conditions, storage roots of cassava may contain high levels of cyanogenic glycosides which must be removed by processing prior to consumption. It is generally consumed boiled or fried as fresh "table" cassava or in dried processed forms. The major processed forms of the cassava storage root fall into 4 general categories: meal, flour, chips and starch. Meal forms include *gari* and *farinha* (figure 1.2.1) and form the bulk of processed cassava consumed in the tropics. Cassava chips and starch are used primarily for animal feeds or as a raw material for starch based industries; although processed cassava chip forms, such as *abacha* in Nigeria, and starch forms such as *tapioca* (*sago*) are also popular human foods (reviewed in Onwueme 1978).

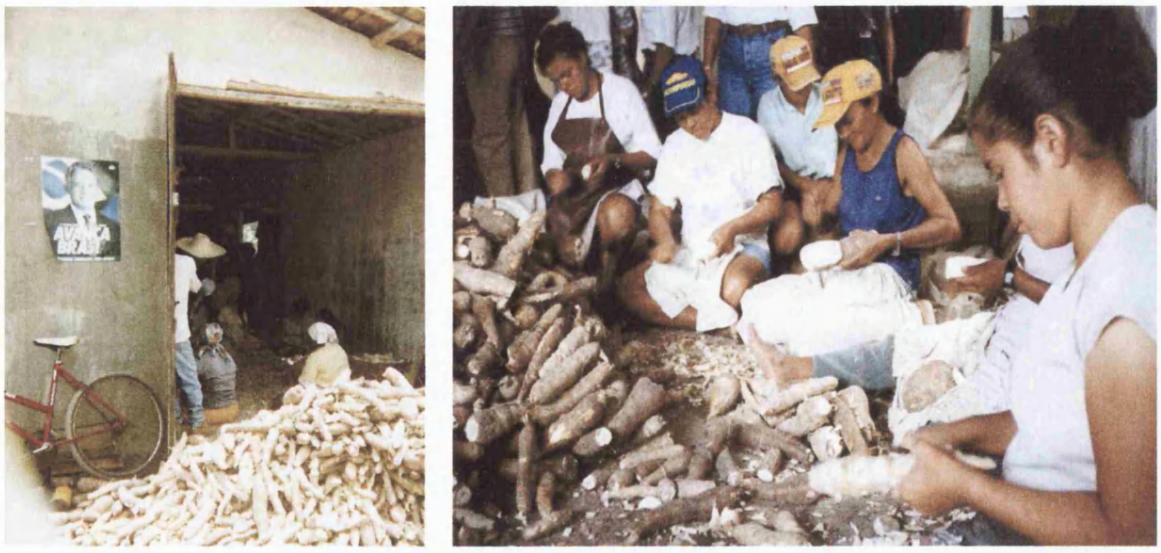


Figure 1.2.1 Preparation of *farinha* in north eastern Brazil, August 1998. Freshly harvested roots are peeled, grated, mechanically pressed, sieved and toasted for several hours in a heated rotating oven. It is consumed dry as an accompaniment to other food or mixed with hot or cold water to form a paste.

1.3 Topics in cassava research

The FAO has identified several key areas for cassava research including yield; pest and disease resistance; cyanide and dry matter (starch and protein) content; and post harvest storage (Wenham 1995).Two centres within the Consultative Groups on International Agricultural Research (CGIAR) system were set up in 1968. These centres - CIAT (Centro International de Agricultura Tropical) located at Cali, Colombia and IITA (International Institute of Tropical Agriculture) located at Ibadan, Nigeria, were mandated to improve the quality and quantity of basic food commodities in the tropics and have been instrumental in the breeding and introduction of high yielding cassava varieties. Cassava production has doubled in Africa and Asia in the last 20 years (1970 - 1996) from 40.5 to 83.2, and 23.2 to 46.3 million metric tonnes per annum respectively (FAO 1998). The Cassava Biotechnology Network (CBN) was set up in 1988 to facilitate communication and collaboration among cassava researchers. The first objectives of the CBN were to enlist advanced laboratories for cassava biotechnology research around a common strategic agenda, in order to use existing research investment cost- effectively and to stimulate relevant research in cassava-growing countries (Thro and Fregene 1998). As a result significant progress has been made in cassava research over recent years.

1.3.1 Pests and diseases

Despite its relative tolerance, pests and diseases can cause considerable losses in cassava yield and can result in total losses in some areas (Puonti-Kerlas 1998). Insect pests include cassava mealy bug (*Phanacoccus manihoti*), whitefly (*Bemisia tabaci*), green and red spider mites (*Mononychellus tanajoa, Tetranychus telarius*) and root knot nematodes (*Meloidogyne incognita*). Major viral diseases are cassava common mosaic virus (CsCMV), East African cassava mosaic virus (EACMV) and African cassava mosaic virus (ACMV). ACMV is transmitted by whitefly (*Bemisia tabaci*) and by infected tools and planting materials and can cause local loss of the entire crop. In 1998, a severe pandemic associated with a recombinant virus, affecting Uganda and western Kenya occurred and resulted in severe shortages and famine in areas where the crop formed the major staple. The novel virus (EACMV/Ug) has arisen from a recombination between ACMV and EACMV (Pita 2000). The major microbial disease is cassava bacterial blight, caused by *Xanthamonas campestris* pv *manihotis*. The accumulation and spread of these and other systemic diseases is exacerbated by the method of vegetative propagation. Sanitation techniques which have been developed

include propagation from seeds and meristem tip culture (Frison 1994, Iglesias *et al.* 1994). Research has been facilitated by the establishment of core germplasm collections and the deployment of molecular tools. A molecular genetic map with markers linked to resistance genes to ACMV and cassava bacterial blight has been developed at CIAT (Fregene *et al.* 1997) and conventional breeding programmes have been carried out to produce resistant cultivars (Puonti-Kerlas 1998). Genetic engineering approaches, for example transgenic plants expressing the coat protein gene of CsCMV have been developed and the plants are now undergoing trail to determine whether the transgene confers elevated resistance (Schopke *et al.* 2000). A cell suspension system has been used to study biochemical components of disease resistance, such as the oxidative burst and induction of defence related genes in response to elicitation (Gomez Vasquez 1998).

1.3.2 Cyanide and dry matter content

Both the leaves and storage roots of cassava are cyanogenic, i.e. cyanohydrin is produced by the action of a β-glucosidase enzyme, linamarase, on the cyanogenic glycosides linamarin and lotaustralin following tissue damage. Subsequently cyanohydrin is converted to acetone and HCN (hydrogen cyanide) either spontaneously or catalysed by the enzyme hydroxynitrile lyase. Unprocessed cassava roots may contain 15 - 1500 mg / Kg cyanide equivalents (Puonti-Kaerlas 1998) which must be removed by processing such as removal of the outer layer, soaking, boiling and draining prior to use. Long term exposure can lead to neurological disorders such as tropical neuropathy and the disease Konzo found in eastern central Africa. Cassava genes for the cyanogenic enzymes linamarase and hydroxynitrile lyase have been cloned and characterised (Hughes and Hughes 1994, Hughes *et al.* 1998). Transgenic approaches involving increased expression of the endogenous hydroxynitrile lyase gene under control of a constitutive CaMV 35s promoter are underway (Arias-Garzon and Sayre 2000).

Cassava storage roots have low protein content and the quality of protein present is poor due to low composition of the amino acids methionine, lysine, tryptophan, phenylalanine, tyrosine and cysteine. Consequently, rural communities that rely heavily on cassava diets usually have a high incidence of protein malnutrition. Two types of approaches have been suggested. The first is to accept that cassava storage roots are a supplier of dietary carbohydrate and little else, and alternate foods should be used to supply protein (Onwueme 1978). The second is to attempt to breed cassava cultivars

that are high in protein. The protein content of cassava roots is often high before root filling occurs; hence varieties that are harvested early or that are inherently low yielding tend to have higher protein levels. However selecting for high protein content may eventually lead to a lower yield potential because the synthesis of protein requires almost twice as much of the primary products of photosynthesis as the synthesis of a similar weight of starch (Cock 1985). Although potential does exist to increase vitamin A (Nair *et al.* 1996, Iglesias *et al.* 1997), and protein content to a certain extent, where farmers have access to markets increased income from cassava sales resulting in diversified diets would be preferred by farmers as a way to achieve better nutrition (Thro *et al.* 1997). Cassava leaves are highly nutritious, and richer than the root in protein and vitamins (typical composition per 100g fresh weight is 6g protein, 1g fat, 0.2g calcium, 0.3g iron, 0.2mg vitamin B_1, 0.3mg vitamin B_2, 200mg vitamin C, 10,000 IU vitamin A and 1.5mg niacin). Although the leaves also contain cyanogenic glycosides, these can be removed by chopping and boiling the leaves, which may be incorporated into stews or broths as is prevalent in Sierra Leone and the Congo basin (Onwueme 1978, Woyinka *et al.* 1995).

1.3.3 Post harvest storage

Cassava storage roots undergo a rapid post-harvest deterioration within 24 to 72 hours after harvest, which renders them unpalatable and unmarketable for consumption or industrial uses such as starch extraction. The deterioration can be divided into 2 stages. The first, is referred to as primary or post-harvest physiological deterioration (PPD), and is an endogenous process characterised by blue/black discoloration of the xylem vessels, often developing from wound sites resulting from harvest (Averre 1967, Noon and Booth 1977). Subsequently, secondary or microbial deterioration occurs. Organisms commonly associated with secondary deterioration of the cassava storage root include *Rhizopus* sp., *Bacillus* sp., *Penicillium* sp., *Trichoderma harzianum, Aspergillus niger, Aspergillus flavus, Lasiodiploidia threobromae, Cylindrocarpon candidum, Rigidoporus lignosis, Phythophthora drechsleri, Botryodiploidia threobromae* and *Pythium butleri* (Booth 1976, Taniguchi *et al.* 1984, Wenham 1995).

Subsistence farmers respond by keeping the crop in the ground until required or by processing the roots into a storable dry product. With increasing urbanisation however, there is an impetus for enhanced storability of cassava. Due to storage problems, transport and distance, good quality fresh cassava is scarce in urban centres thereby encouraging import of carbohydrate alternatives such as wheat. As a result cash income

to small national farmers is decreased (Wheatley and Best 1991). In addition, development of small and larger scale processors of cassava for starch and other industrial processes is impeded. Thus, enhanced storability of cassava is seen as a priority (Wenham 1995, Beeching 1998).

1.3.4 Other aspects – genome and genetic resources, genetic transformation, micropropagation and farmer participatory research

A number of molecular tools have been developed which may help to guide breeding decisions for crop improvement. A molecular genetic map, markers linked to disease resistance genes and marker aided studies of complex traits such as post-harvest storage now exist or are being developed for cassava (Fregene *et al.* 1997, Sanchez *et al.* 1999, Fregene *et al.* 2000). Existing diversity in the cassava core collection with respect to traits such as β–carotene content, PPD and disease resistance have been evaluated (Iglesias *et al.* 1996, Iglesias *et al.* 1997, Sanchez *et al.* 1999); and diversity within primary, secondary and teriary gene pools (such as *M.esculenta* subsp. *M.flabellifolia*, *M.glaziovii* and *M.dichotoma*), which may contribute as gene suppliers in breeding programmes have been investigated (Allem *et al.* 2000a, Allem *et al.* 2000b). A geographical information system (GIS) to characterise wild genetic resources and landraces that may show site specific traits regarding biotic and abiotic stresses has been developed. Germplasm accessions having both a known place of origin correlated with prevailing environmental conditions may contribute towards identification of genotypes adapted to particular environmental stresses such as soil salinity, low fertility or temperature (Burle *et al.* 2000).

With regard to non-traditional breeding techniques, transformation systems for cassava based on both particle bombardment and *Agrobacterium* mediated transformation systems have been developed (Gozalez *et al.* 1998, Schopke *et al.* 1996, Schopke *et al.* 2000).

Micropropagation (*in vitro* culture) techniques have been instrumental for maintaining germplasm collections and for treating and multiplying disease free planting material for distribution to farmers (Ibrahim *et al.* 1998, Woodward *et al.* 2000, FAO 2000).

Farmer participatory research allows a linking between researchers and farmers to ensure a genuine need, and participatory projects within CBN have been initiated since 1996 (Thro 1998). As noted by Fakuda (1998), many cassava varieties developed have not been adopted, and this may be attributed to the absence of farmer participation in the process of identifying priorities and generating technologies; as well as the absence of

feed back mechanisms to researchers about criteria used by farmers to decide whether to adopt or disregard a particular technology or variety. For example, although cassava cyanogenesis is often identified as a research priority in northern laboratories, growers have long been well aware of processing procedures required to remove cyanogenic glycosides. In many regions "bitter" varieties are highly prized for their flavour, low "theftability" and reduced spoilage by animals – all of which are directly related to the high HCN equivalents (Rosling 1998). Cassava toxicity occurs in a context of poverty, and toxicity effects occur where there is a low sulphur (and sulphur containing amino acid) content in the diet; and where people are forced to subsist primarily or exclusively on cassava allowing a high internal dose. Thus solving cassava toxicity problems is not directly within the scope of molecular biology but should be tackled by improving the economic and living standards of poor cassava farmers (Rosling 1998, Essers 2000).

1.4 Post-harvest Physiological Deterioration (PPD) of the storage root

Primary or post-harvest physiological deterioration (PPD) is characterised by blue/black "vascular streaking" of the xylem vessels of the cassava storage root, followed by discoloration of the storage parenchyma (figure 1.4.1). It is associated with a bitter unpalatable flavour and alteration in starch grain structure. PPD often develops from wound sites and has been shown to be an endogenous root disorder, rather than a microbially mediated deterioration process, although it is often followed by secondary microbial deterioration. It has been studied at the biochemical and cytological level for several years but remains poorly understood (Puonti-Kerlas 1998). Many of the metabolic changes which occur during PPD resemble those of a normal plant wound response reaction, and it has been proposed that the cause of PPD may be a sustained wound reaction spreading systemically from the wound site to the whole root (Beeching et al. 1994, 1995). Other authors have proposed that PPD may reflect a wound induced senescence response (Lalaguna and Agudo 1989, Passan and Noon 1997). With the construction of a PPD related cDNA library in this laboratory (Beeching *et al.* 1997, Y.Han 2000) the analysis of processes occurring during PPD at a molecular level has recently become possible.

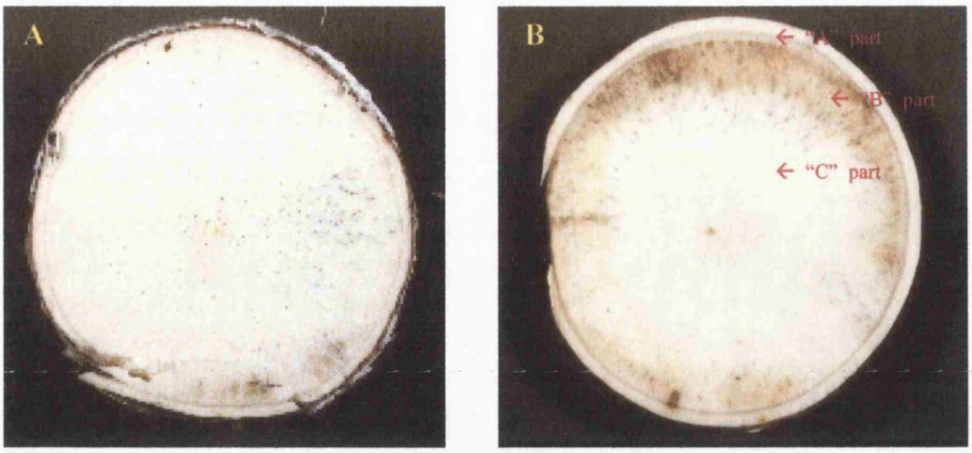


Figure 1.4.1 Post-harvest physiological deterioration (PPD) of the cassava storage root. Panel A shows a storage root exhibiting initial blue/black vascular streaking. Panel B shows a more advanced stage of PPD, with vascular streaking and tissue browning clearly evident. Uritani and colleagues (1983) subdivide the parenchyma part of the root in to the "A" part of the parenchyma just underlying the cortex, which commonly shows few symptoms of PPD, the "B" or intervening part which shows most pronounced symptoms, and the "C" or internal part which shows less development of PPD. The regions of parenchyma tissue corresponding to these parts are indicated by arrows in panel B.

1.4.1 Variation in susceptibility

Susceptibility of cassava storage roots to PPD shows both genetic and environmental components. In a study of available genetic variability among a group of elite cassava clones grown in different ecosystems in Colombia, genetic variability on the last day of evaluation accounted for 52% of the observed variability, indicating the possibility of progress in a genetic selection programme (Iglesias *et al.* 1996). Defoliation as a result of biotic or abiotic stresses has been observed to reduce root susceptibility to PPD and it has been observed that non locally adapted cultivars were less susceptible to PPD than local ones (Wheatley 1980). Pruning of the plants by removal of the top of the plant 20-30cm above the base of the stem two to three weeks prior to harvesting has also been shown to reduce susceptibility of the roots to PPD (Data *et al.* 1984, Kato *et al.* 1991, Tanaka *et al.* 1984, Hirose *et al.* 1984). The similarity in effects of these stresses on root susceptibility to PPD suggest that pre-stressing may prepare the roots for further stress perhaps in a way somewhat analogous to SAR (systemic acquired resistance). Storage roots of different developmental stages have been investigated, and it has been demonstrated that development of PPD is not correlated with root age (Hirose and Data 1984).

1.4.2 Post harvest storage techniques

A number of post harvest storage techniques have been developed which can inhibit the occurrence of PPD, however these are often impractical or uneconomical. Storage of the roots in a polythene bag or dipping in wax can effectively prevent PPD and enable the roots to be stored for more than two weeks, particularly if the roots are pre-treated with a fungicide (thiobendazole based) to control microbial deterioration. Roots with minimal damage that are packed within 3 hours after harvest can be stored for up to 15 days (Wheatley *et al.* 1989). Roots dipped in paraffin wax at 90-95°C for periods as short as 45 seconds can be stored for 1 to 2 months and this method is commercially used, particularly for export (Ravi *et al.* 1996). Freshly harvested roots may be stored for 2 to 4 weeks in acceptable condition for marketing in boxes packed with moist sawdust (Booth 1976). Roots dipped in 1% benomyl and stored in a polythene lined cardboard box, with either moist sawdust or sand or a mixture of both, could be stored for up to 13 weeks (Wickham and Wilson 1988). Curing, that is treating of the roots at high temperature (35°C) and humidity (80-85% RH) for a period of 2-4 weeks could extend the storage time for a period of up to 4 weeks (Booth 1976). Although these methods are effective in prolonging the shelf life of cassava, subsequent injury of the roots results in a PPD response similar to that of fresh roots (Ravi *et al.* 1996).

Many of the above methods may function by either artificially sealing the root (wax treatments), and/or by providing suitable conditions (high temperature and humidity) to allow formation of a wound periderm (curing, storage in polythene bags etc). Wound periderm formation in injured cassava roots does occur under conditions of high temperature and humidity, although it is slower than observed in other root crops (Ravi *et al.* 1996).

A number of temperature treatments can inhibit the response. Cassava roots may be stored at low temperature 0-5°C for several weeks (Montaldo 1973, Booth 1976), however PPD developed within 1-2 days if the roots were transferred to 24°C. Dipping of roots in hot water at 60°C for 45 minutes could also inhibit PPD over a 5 day observation period (Averre 1967).

1.4.3 Previous studies on the cytology and biochemistry of PPD

Evidence accumulated during the 1970s to indicate that PPD was an endogenous physiological process, rather than a microbially mediated one, since no micro-organisms could be isolated from the margins of discoloured tissue (Averre 1967, Booth 1976). Inoculation of roots with isolates from microbially rotted roots could not induce the

early symptoms of vascular streaking (Noon and Booth 1977). In addition, surface sterilization of harvested roots with sodium hypochloride (Averre 1967) or treatment of the roots with fungicides (dicloran and benomyl, in concentrations up to 20,000ppm) and bactericides (streptomcycin sulphate 2.5%) could not inhibit the reaction (Noon and Booth 1977). In contrast, relatively crude treatments such as dipping the roots in hot water (60°C) for 45 minutes could completely inhibit PPD over a storage period of 5 days (Averre 1967), leading the authors to suggest that the nature of PPD was enzymatic in origin. Cassava storage roots are inevitably injured during harvest and subsequent handling and transport can cause further damage. Studies carried out during the 1970s indicated that PPD developed from wound sites and increased mechanical damage was associated with increased symptoms of PPD (Booth 1976). A correlation between water loss and PPD was suggested since severe mechanical damage resulted in both a higher degree of fresh weight loss and increased symptoms of PPD. When transverse root slices were stored with one end covered with PVC film, vascular streaking initiated at the uncovered surface (Booth 1976). When stored at low humidity (45-55% RH) wounded surfaces developed a dry white layer within 72 hours, beneath which a greenish brown band developed and vascular streaking was observed along the xylem tissues. When roots were stored at high humidity (80-90% RH) a wound periderm was formed and vascular streaking was inhibited (Marriot *et al.* 1978, Rickard 1985).

Microscopic observations revealed the formation of coloured occlusions from the xylem parenchyma which moved into adjacent xylem vessels via pit areas (Rickard *et al.* 1979, Rickard 1983). The main components of the xylem occlusions were carbohydrates, lipids and lignin-like materials thought to be condensed tannins resulting from the polymerisation of leucoanthocyanidins and catechins. An increase in phenolic components was also noted.

A marked increase in respiration rate occurred in response to injury, with a 2 fold increase occurring within 1 day after harvest. Roots that were severely damaged by removal of the periderm or cortex showed higher respiration rates than intact roots (Data *et al.* 1984, Hirose *et al.* 1986). Roots from pruned plants, which show reduced susceptibility to PPD, showed lower respiration rate than roots from non pruned controls (Data *et al.* 1984).

Increased levels of ethylene occurred in cassava roots prior to the onset of PPD. Ethylene was found to increase dramatically after a lag of around 6 hours after injury and continued to increase over a 22 hour period (Plumbley *et al.* 1981, Hirose *et al.*

1984a). In addition, higher levels of ethylene were found in roots that were more susceptible to PPD, although pre-harvest pruning did not prevent the synthesis of ethylene in the root after injury (Hirose *et al.* 1984b, Hirose *et al.* 1986). When cut tissue blocks were examined, highest levels of ethylene production occurred in the cortical parenchyma, whilst the storage parenchyma showed lower levels (Hirose *et al.* 1984a,b). These data suggest that ethylene may play a role in signal transduction during PPD. In other plant systems the plant hormone ethylene plays a coordinating role in wound responses, senescence and fruit ripening.

Studies on enzyme activities during PPD have indicated increased levels of phenylalanine ammonia lyase (PAL), catalase, peroxidase, polyphenol oxidase and acid invertase (Czyhrinciw and Jaffe 1951, Rickard 1981, 1985, Uritani *et al.* 1984, Plumbley *et al.* 1981, Padmaja and Balagopal 1985). The activity of phenylalanine ammonia lyase (PAL), the key entry enzyme into phenylpropanoid biosynthesis, peaked within 40 hours after injury, coinciding with the increase of phenolic compounds produced during PPD (Tanaka *et al.* 1983, Uritani *et al.* 1983). Levels of peroxidase, acid invertase and PAL were lower in roots from pruned plants, although pruning had no significant effect on polyphenol oxidase activity (Data *et al.* 1984, Tanaka *et al.* 1984, Kato *et al.* 1991).

Changes in membrane structure during PPD have shown a progressive decline in phospholipid content, indicating membrane degradation (Sakai *et al.* 1986, Lalaguna and Agudo 1989) and it has been proposed that such membrane breakdown could allow contact between substrates and enzymes resulting in the formation of coloured products observed as vascular streaking.

Intense blue/white fluorescence, due to accumulation of the fluorescent coumarin components scopolin, scopoletin and esculin was observed in the storage parenchyma prior to the onset of PPD (Tanaka *et al.* 1983). Fluorescent components occurred primarily in the "B" part of the parenchyma, that is the intervening part of the parenchyma where symptoms of PPD are first observed. Using TLC, HPTLC and HPLC techniques, the coumarin components were identified as scopolin, scopoletin and esculin. Two flavenoid components that also accumulated during PPD were identified as (+) catechin and gallocatechin (Uritani *et al.* 1984). Scopoletin accumulated first, with a peak around 20 hours after injury, whilst scopolin and esculin peaked around 40 hours after injury (Tanaka *et al.* 1983). Levels of the coumarin components scopolin, scopoletin, and esculin were again found to be lower in roots from pruned plants (Tanaka *et al.* 1984, Wheately and Schwabe 1985).

There is considerable evidence that the coumarin, scopoletin, may play a pivotal role in the development of PPD. Scopoletin accumulates in the cassava storage root with a peak occurring at around 20 hours after injury, followed by a decrease which coincides with the onset of tissue deterioration, suggesting this component is being utilised during the PPD response (Wheatley 1982). In addition, exogenous application of scopoletin ($500mg/dm^3$) to cassava root slices could induce significant vascular streaking within 18 hours. A range of other phenolic compounds such as p-coumaric acid, cinnamic acid, caffeic acid, esculetin and catechol were relatively inactive. Roots from pruned plants responded as vigorously to applied scopoletin as control roots, however if roots were cured for a period of 7 days they showed little or no response to application of scopoletin (Wheatley and Schwabe 1985). Recently, fluorescence microscopy studies have allowed localisation of the fluorescent coumarin compounds produced in the cassava root after injury at the microscopic level (Buschmann *et al.* 2000c). These studies indicate that within 6-12 hours after harvest fluorescence is first detected around the xylem vessels and later spreads throughout the root parenchyma by 48 hours after injury.

Post-harvest deterioration has been demonstrated to be an active process that requires protein synthesis, since treatment with cycloheximide could prevent the appearance of the blue/white fluorescence associated with coumarin accumulation; and could prevent the occurrence of PPD (Uritani *et al.* 1984). In addition, experiments involving *in vivo* labelling of proteins in cassava root disks indicate a massive synthesis of proteins, including novel proteins, as a response to wounding (Beeching *et al.* 1995), although cassava root storage proteins (of 23, 30, 73 and 57KDa) have been reported to decrease during the post-harvest period (Uritani *et al.* 1992).

1.4.4 Molecular biology of PPD

Huang and colleagues (2000) used a cDNA-AFLP approach to characterise 70 transcript derived fragments (TDFs) that showed up-regulated, down-regulated or transient expression during PPD. Based on the sequence homology of these isolated TDFs they identified processes which may occur during PPD as unknown 28%, metabolism 24%, stress/wounding 22%, signal transduction 12%, development 8%, programmed cell death (PCD) 6%.

Recent studies in this laboratory have allowed the construction of a PPD related cDNA library and the isolation and characterisation of cDNA clones known to be involved in wound responses in other plant systems (Beeching *et al.* 1997, Han 2000, Li *et al.*

2000). A cassava cDNA clone and its cognate genomic clone encoding ACC oxidase, the last enzyme in ethylene biosynthesis, have been isolated and are being studied using antisense approaches to further elucidate the role of ethylene in PPD (Li *et al.* 2000). Two cDNAs encoding PAL, designated cMePAL1 and cMePAL3 have been isolated and characterised. MecPAL1 shows transient expression within 8 hours after injury in the cassava storage root (Han 2000). Components of wound repair and defence in other plant systems – a hydroxyproline rich glycoprotein (cMeHRGP) and a β-1,3 glucanase (cMeGLUC) have also been isolated, indicating that components of wound repair are expressed in the cassava root undergoing PPD. Northern analysis indicated cMeGLUC was expressed 72 hours after injury (Han 2000), similarly cMeHRGP transcript did not accumulate to high levels until 72 hours after injury (Han *et al.* 2000). At this time the PPD response was pronounced throughout the root, providing support for the hypothesis that the symptoms of PPD result from inadequate wound repair (Beeching *et al.* 1997). Recently two subtracted cDNA libraries corresponding to early PPD response cDNAs (RNA from 3, 6 and 12 hours after injury used as tester cDNA), and late response cDNAs (RNA from 24, 48 and 96 hours after injury used as tester cDNA), have been constructed in λZAP (Beeching and Reilly, unpublished results) and should allow isolation and characterisation of additional PPD specific cDNAs.

1.5 Experimental strategy

Oxidative processes are known to play a role in wound, senescence and defence reactions in other plant systems (for review see Thompson *et al.* 1986, Baron and Zambryski 1995) and several lines of evidence suggest that discoloration reactions during PPD represent an enzymatically mediated oxidative process.

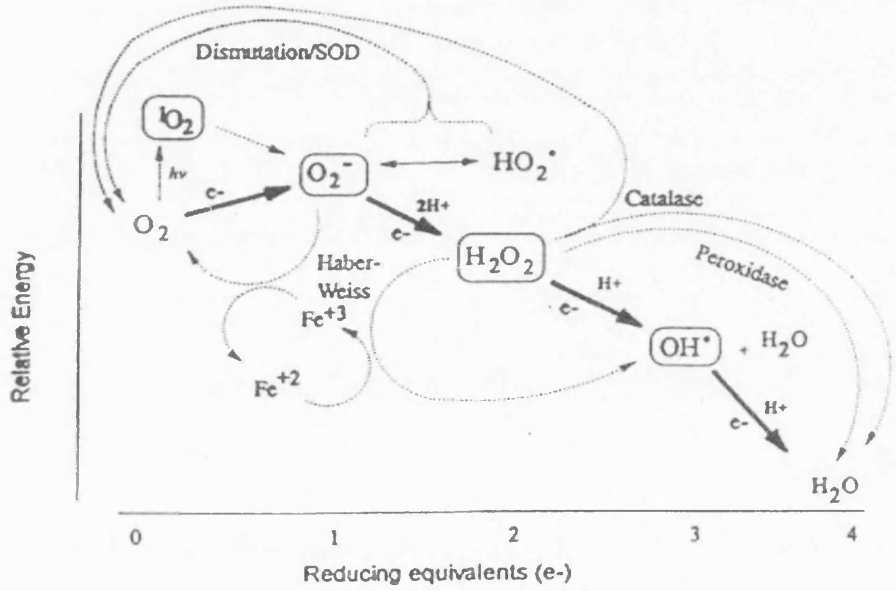
Several earlier studies have indicated that molecular oxygen is required for the development of PPD. Averre (1967) found that roots stored under water or under anaerobic conditions did not develop PPD. Similarly Noon and Booth (1977) found that storage under anaerobic atmospheres including pure CO_2, propane gas or oxygen depleted air could inhibit PPD. Richard (1982) found that storage of roots under 100% nitrogen gas could completely inhibit the development of PPD, whilst storage under nitrogen gas containing various amounts of oxygen (1-10%) led to increased symptoms of PPD with increasing oxygen concentrations.

Marriot *et al.* (1977) suggested that injury results in increased availability of oxygen to internal root tissue, and many of the storage techniques discussed in section 1.4.2 may be effective by exclusion of oxygen. Tanaka and colleagues (1983) suggested that PPD

may result from access of oxygen to internal root tissues via wound sites and xylem vessels followed by enzymatic oxidation of phenolic compounds mediated by peroxidases or polyphenol oxidases, whilst Wheatley and Schwabe (1985) suggested that the components of this reaction might be peroxidase catalysed oxidation of scopoletin to an unknown intermediate.

In addition, a decrease in non-enzymatic antioxidants such as β-carotene and ascorbate during PPD has been reported (Gloria and Uritani 1994, Czyhrinciw and Jaffe 1951), whilst recent results (Iglesias *et al.* 1995, Adewusi and Bradbury 1993) suggest that roots with a high carotene content are less susceptible to PPD.

A schematic representation of reactive oxygen species derived from molecular oxygen and likely inter-conversion pathways thought to exist in plants is shown in figure 1.5.1.



Catalase:

$2H_2O_2 \rightarrow 2H_2O + O_2$

Peroxidase:

$H_2O_2 + 2AH \rightarrow 2H_2O + 2A$

Superoxide Dismutase:

$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

Figure 1.5.1 Reactive oxygen species derived from molecular di-oxygen and likely inter-conversion pathways thought to exist in plant systems (after Baker and Orlandi 1995). Enzyme catalysed inter-conversion reactions are indicated at the base of the figure.

Molecular oxygen is itself relatively un-reactive, however its ease of conversion to more reactive partially reduced forms necessitates a battery of cellular defences - ranging from specific enzymes such as catalase, peroxidase and superoxide dismutase, to non enzymatic antioxidants and quenchers – in order to maintain cellular homeostasis. Where the balance between production and scavenging of ROS shifts, either due to enhanced production or decreased scavenging capability, oxidative stress occurs and can result in cellular damage. The strategy of this project therefore, was to examine the expression of the primary ROS scavenging enzymes catalase, peroxidase and superoxide dismutase during PPD of the cassava storage root, and in addition to examine the production of reactive oxygen species following wounding. The role of such scavenging enzymes and of ROS in other plant systems is discussed more fully in the relevant chapters.

Figure 1.5.2 Storage of cassava storage roots under field conditions at CIAT, Cali, Colombia

1.6 Aims of the project

The molecular mechanisms underlying PPD remain poorly understood, however the PPD response may involve enzymatically mediated oxidative processes. The aim of this project therefore was to utilize a PPD related cDNA library, previously constructed in this laboratory, to generate probes which could be used to study the expression of ROS scavenging enzymes during the post-harvest period, and in a range of cultivars showing differing susceptibility to PPD. In addition, any cDNA clones generated could be transferred to CIAT for inclusion on the cassava genetic map. In order to elucidate some of the signalling pathways which may lead to PPD, the production of ROS as a response to injury, and the expression of genes encoding ROS scavenging enzymes in response to pre-harvest pruning, ethylene and methyl jasmonate was also examined.

CHAPTER TWO:
MATERIALS AND METHODS

2.1 Bacterial strains:

Escherichiae coli NM514

hsdR514 ($r_k^- m_k^+$) *argH galE galx strA lycB7* (Hfl^+)

Recombination deficient strain suitable as a host for phage λgt10 and used for cDNA library construction. NM514 is a *lycB7* restriction deficient derivative of POP101. The *lycB7* mutation confers high frequency of lysogeny genotype (Hfl^+). The use of this strain allows stringent biological selection of recombinant phage since non-recombinant phage are forced into a lysogenic pathway.

Escherichiae coli DH5α

ϕ80d*lac*ZΔM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17($r_k^- m_k^+$), *sup*E44, *rel*A1, *deo*R, Δ (lacZYA-*arg*F) U169

A recombination deficient strain used for plating and growth of plasmids. The ϕ80d*lac*ZΔM15 allows α complementation with the amino terminus of β-galactosidase encoded in pUC and pBluescript vectors. Strains such as DH5α are preferred for production of plasmids for fluorescent sequencing since they are $endA^-$ and thus lack high levels of nuclease activity.

Escherichiae coli JM109

[F', *tra*D36, *pro*AB, $lacI^q$ Z ΔM15]

*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 ($r_k^- m_k^+$), *rel*A1, *sup*E44, Δ(*lac-pro*AB)

A recombination deficient strain used for plating and growth of plasmids. Suitable for α complementation with the amino terminus of β-galactosidase encoded in pUC, pGEM and pBluescript vectors. The F episome is required for blue/white colour selection. To maintain the F episome the strain should be maintained on M9 plates supplemented with 1mM thiamine-HCl.

2.2 Phage and plasmid vectors

2.2.1 Phage λ vectors:

λgt10 — A 43.34kb phage vector suitable for cDNA library construction and subsequent hybridisation screening with nucleic acid probes, since large plaques of uniform size are produced. It has a cloning capacity of 7.6kb and allows biological selection of recombinants by insertion disruption at a unique *Eco*RI site within the cI repressor gene. Parental phage with active cI repressor favour the lysogenic pathway, whilst recombinant phage producing inactive cI repressor favour the lytic pathway producing clear plaques. A vector map and a diagrammatic representation of the *Eco*RI adaptors used for cDNA cloning are shown in figure 2.2.1.

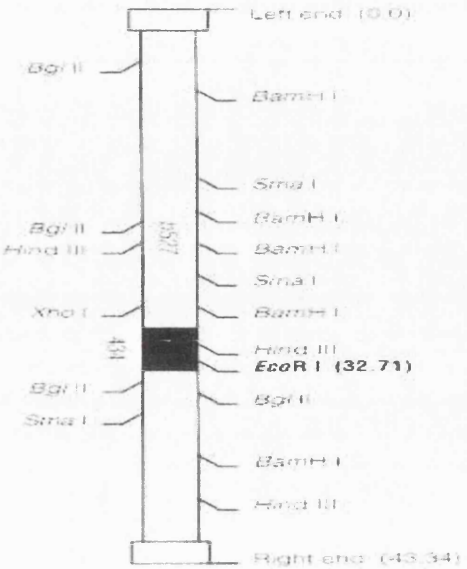
2.2.2 Plasmid vectors:

pBluescript II (KS -) — A 2.96 kb standard cloning vector conferring ampicillin resistance to host cells. Suitable for blue/white colour screening and *in vitro* RNA transcription. A plasmid map is shown in figure 2.2.2a.

pUC18 — A 2.69 kb cloning vector conferring ampicillin resistance to host cells. Suitable for blue/white colour screening. A plasmid map is shown in figure 2.2.2b.

pGEM T-easy — A 3.02kb linearised cloning vector from Promega suitable for ligation and sub-cloning of PCR products. The plasmid confers ampicillin resistance to host cells. Suitable for blue/white colour screening and *in vitro* RNA transcription. A plasmid map is shown in figure 2.2.2c.

A



B

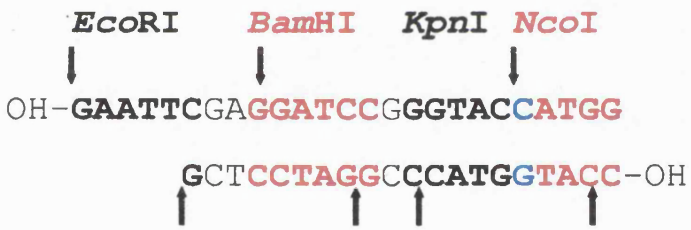


Figure 2.2.1 Phage lambda vector λ gt10.
A 43.34kb phage vector suitable for cDNA library construction and hybridization screening with nucleic acid probes. For library construction, cDNA inserts ligated to *Eco*RI adaptors are inserted at the unique *Eco*RI site, disrupting the cI gene. Panel A = λ gt10. Panel B = *Eco*RI adaptor, restriction enzyme recognition sites are shown in bold or red, nucleotides contributing to overlapping recognition sites (*Kpn*I + *Nco*I) are shown in blue, cleavage sites are indicated by arrows.

Figure 2.2.2a Plasmid pBluescript.
A 2.96kb standard cloning vector. The vector incorporates a multiple cloning site; a β-lactamase (*bla*) gene encoding Amp^R; T7 and SP6 RNA polymerase promoters and a *Lac* Z region encoding the *Lac* Z α peptide. The vector may be used for standard cloning procedures and may also be used for *in vitro* transcription. The *Lac* Z region allows selection of recombinants using blue/white colour screening.

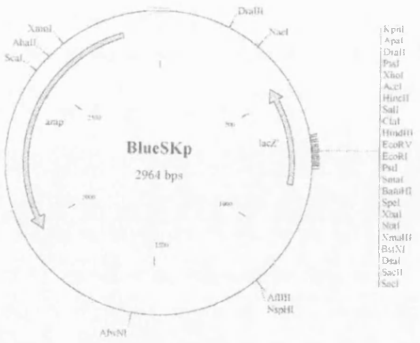


Fig 2.2.2b Plasmid pUC18.
A 2.69 kb cloning vector. The vector contains a multiple cloning site; a β-lactamase (*bla*) gene encoding Amp^R; and a *Lac* Z region encoding the *Lac* Z α peptide. The vector may be used for standard cloning procedures. The *Lac* Z region allows selection of recombinants using blue/white colour screening.

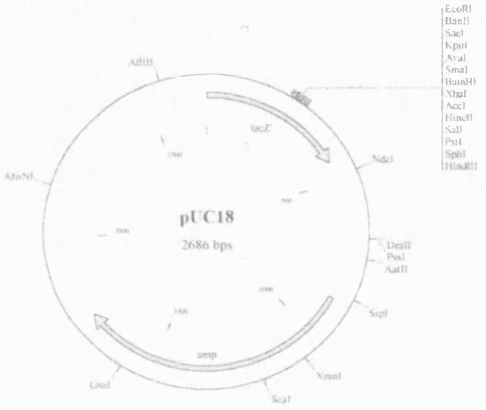
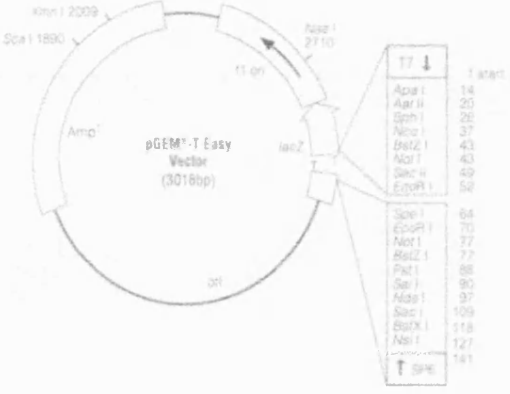


Figure 2.2.2c plasmid pGEM-T easy.
A 3.02kb linearised cloning vector from Promega. The vector incorporates a multiple cloning site; a β-lactamase (*bla*) gene encoding Amp^R; T7 and SP6 RNA polymerase promoters and a *Lac* Z region encoding the *Lac* Z α peptide. The vector may be used for PCR cloning and may also be used for *in vitro* transcription. The *Lac* Z region allows selection of recombinants using blue/white colour screening.



2.3 Heterologous probe DNAs:

Plasmid name	Vector and / or cloning site	Insert
pCAT 1	pBluescript *Xho*I / *Xba*I	*Nicotiana plumbaginifolia* (curled leaf Tobacco) catalase cDNA CAT1
pCAT 2	pBluescript *Xho*I / *Xba*I	*N. plumbaginifolia* catalase cDNA CAT2
pCAT 3	pBluescript *Bam*HI / *Kpn*I	*N. plumbaginifolia* catalase cDNA CAT3
pSOD1	pUC18 *Pst*I	*N. plumbaginifolia* Mn superoxide dismutase cDNA SOD1
pSOD2	pUC18 *Pst*I	*N. plumbaginifolia* Fe superoxide dismutase cDNA SOD2
pSOD3	pUC18 *Pst*I	*N. plumbaginifolia* Cu/Zn superoxide dismutase cDNA SOD3
GPX	pBluescript *Eco*RI	*Nicotiana sylvestris* (wood Tobacco) glutathione peroxidase cDNA
ELI 11	pUC 9 *Eco*RI	*Petroselenum crispum* (Parsley) anionic peroxidase cDNA
pAP3	pUC 19 *Eco*RI	*Solanum tuberosum* (Potato) anionic peroxidase.
pAP4	pUC 19 *Eco*RI	*Solanum tuberosum* (Potato) anionic peroxidase.
PX7	pBluescript KS *Eco*RI	*Cenchrus ciliaris* (Buffel Grass) peroxidase cDNA
Shpx 2	pBluescript SK+ *Eco*RI	*Stylosanthes humilis* (Alfalfa estilosante) peroxidase cDNA
Shpx 6	pBluescript SK+ *Eco*RI	*Stylosanthes humilis* (Alfalfa estilosante) peroxidase cDNA
acidic peroxidase	PUC 18 *Eco*RI	Tobacco peroxidase cDNA

Plasmids pCAT 1, pCAT 2, pCAT 3, pSOD1, pSOD2, pSOD3 and GPX were a gift of M.Van Montagu, University of Gent, Belgium. ELI 11 was a gift from K. Hahlbrock, Max-Planck Institute, Germany. pAP3 and pAP4 were a gift from P. Kolattukudy, Ohio State Biotechnology Centre, USA. PX7 was a gift from R. Birch, University of Queensland, Australia. Shpx 2 and Shpx 6 were a gift from J. Manners, University of Queensland, Australia. The tobacco acidic peroxidase was a gift from K.Lawton, CIBA-GEIGY, USA.

2.4 Plant material:

Cassava (*Manihot esculenta* Crantz) cultivars used:

Cultivar	Female Parent or Common Name	Male Parent	Susceptibility to PPD
MCOL 22	*Uvita* Colombian Landrace		High
SM 985 - 9	CM 2770 - 7		High
CM 2177-2	CM 430-37	CM 840-138	Medium to High
CM 7033 - 3	HMC 1	CM 2563 - 5	Low
MBRA 337	Brazilian Landrace		Low
MDOM 5	Dominican Republic Landrace		Low
MPER 57	Peruvian landrace		Low
MNGA 1	TMS 30001		not determined
MVEN 77	*Venezuela* Venezuelan Landrace		Medium
MNGA 2	TMS 30572		Medium

Storage roots were obtained from greenhouse-grown plants at Bath; as air freighted material from CIAT, Colombia; or were harvested and used at CIAT, Colombia. Greenhouse conditions at the University of Bath were 22 - 28 °C, relative humidity (RH) 40 – 80 % and a light period of 14 hours per day.

Roots from CIAT were grown under field conditions at the research centre at Cali-Palmira. In order to prevent or minimise initiation of post-harvest physiological deterioration (PPD) in air freighted roots during transit, roots were dipped in paraffin wax containing the fungicide Mertek (2%) and shipped immediately after harvest. On arrival, the wax was removed and post-harvest physiological deterioration was induced by wounding of the storage roots by removal of the proximal and distal ends which were covered with parafilm and cutting of a "V" shaped incision through the epidermis along the length of the root. Leaf material for genomic DNA extraction was obtained

from greenhouse-grown plants at Bath. Leaf and petiole material for RNA extraction were obtained and processed at CIAT, Colombia.

The cultivar identification code above is the CIAT identification code relating to country of origin, e.g. MCOL 22 denotes Manihot Colombia accession number 22, MNGA 2 denotes Manihot Nigeria accession number 2. Hybrids produced at CIAT are denoted by CM. SM refers to the cultivar accession code used at IITA, Nigeria and retained by CIAT. The PPD susceptibility data were provided by CIAT, Colombia (M.Bonierbale pers. com.).

2.5 cDNA Library: The cDNA library used was constructed in λgt10 by Y.Han (Beeching *et al.*, 1997) using the Amersham Rapid cDNA-cloning module. cDNA inserts were prepared from total RNA isolated from cassava cultivar MNGA 1 storage roots 48 hours after harvest. Following blunt end ligation of λ adapters to both ends of the cDNAs, inserts were subcloned into the unique *Eco*RI site of λgt10.

2.6 Culture media for the growth and maintenance of bacterial strains:

LB media	per litre:
Bacto Tryptone	10g
Bacto Yeast Extract	5g
NaCl	10g

The pH was adjusted to 7.0 before adding agar (1.5% w/v) if required. Made up to 1l and sterilised by autoclaving at 10^5 Nm^{-2} for 20 minutes.

M-9 media	per litre
Na_2HPO_4	6g
KH_2PO_4	3g
NaCl	0.5g
NH_4Cl	1g

The pH was adjusted to 7.4 before adding agar (1.5% w/v). Media was made up to 1l and sterilised by autoclaving at 10^5 Nm^{-2} for 20 minutes. After cooling to 50°C the following filter sterile components were added:

1M $MgSO_4$	2ml

1M $CaCl_2$	0.1ml
20% glucose	10ml
1M thiamine-HCl	1ml

LB - Top agar/agarose

Made up as for LB agar but with agar/ agarose concentration at 0.7% (w/v).

Agarose was used for plates intended for plaque lifts and plate lysates, agar for plates used for library titration. Top agar/ose was used molten at 45°C

M-broth

Made up as for LB media with 20% maltose added after autoclaving to a final concentration of 0.4% (w/v).

20% Maltose

10g maltose dissolved in 50ml of MilliQ water and sterilised by filtration. Stored at 4°C.

NYZCM medium	per litre
Casein Hydrolysate	11g
NaCl	5g
Bacto Yeast Extract	5g
$MgSO_4.7H_2O$	2g

Added to 950ml deionised water. The pH was adjusted to 7.0, the volume adjusted to 1l and media was sterilised by autoclaving at 10^5 Nm^{-2} for 20 minutes.

SOB	per litre
Bacto Tryptone	20g
Bacto Yeast extract	5g
NaCl	0.5g

Added to 950ml deionised water and 10ml of 250mM KCl added. The pH was adjusted to 7.0, the volume made up to 1l and the medium autoclaved at 10^5 Nm^{-2} for 20 minutes. After cooling to <60 °C 5ml of filter sterile 2M $MgCl_2$ was added. For the preparation of selective SOB agar plates supplemented with $MgSO_4$, agar (typeIII technical) was added to 1.5% (w/v) prior to autoclaving, and after cooling to <60 °C, 5ml of filter sterile 2M $MgCl_2$, 20ml of 1M $MgSO_4$, and appropriate antibiotic were added.

SOC medium	per litre
Bacto Tryptone	20g
Bacto Yeast extract	5g
NaCl	0.5g

Added to 950ml deionised water and 10ml of 250mM KCl added. The pH was adjusted to 7.0, the volume made up to 1l, and the medium autoclaved at 10^5 Nm^{-2} for 20 minutes. After cooling to <60 °C, 5ml of filter sterile 2M $MgCl_2$ and 20ml of filter sterile 1M glucose were added.

Selective media

For general growth and maintenance of *E.coli* strains harbouring ampicillin resistance conferring plasmids, ampicillin was added to the media to a final working concentration of 50μg/ml. For selection of transformants, ampicillin was added to the media to a final working concentration of 100μg/ml.

2.7 Experimental methods:

Preparation of reagents and solutions referred to below are described in section 2.8 (Reagents and solutions).

2.7.1 Preparation and transformation of competent *E.coli*

2.7.1.1 Preparation of competent *E.coli*

A modification of the method of Cohen *et al.* (1972) as described in Sambrook *et al.* (1989) was used. A single colony (2-3mm diameter) was picked from a freshly grown plate and used to inoculate 100ml of SOB medium. The culture was grown for ~ 3 hours (OD_{600} = 0.3) in order to ensure that the viable cell number did not exceed 10^8 cells/ml. The culture was transferred to two sterile ice-cold centrifuge tubes and the tubes were stored on ice for 10 minutes. The cells were then recovered by centrifugation at 4000 rpm for 10 minutes at 4°C in an SS34 rotor. The supernatant was poured off and the tubes were placed in an inverted position to allow residual medium to drain. Each pellet was then re-suspended in 10ml of ice cold 0.1M $CaCl_2$, and centrifuged and drained as previously. Each pellet was then re-suspended in 2ml of ice cold 0.1M $CaCl_2$. Using pre-chilled sterile pipette tips the competent cells were dispensed in 200μl aliquots into pre-chilled microfuge tubes and stored on ice. Cells were used immediately or after storage on ice overnight.

2.7.1.2 Transformation of competent *E.coli*

Up to 50ng of plasmid DNA or 1µl of ligation mix was added to 200µl of competent *E.coli* cells and gently stirred using a sterile pre-chilled pipette tip. In order to allow estimation of transformation frequency, a known amount of super-coiled plasmid was used to transform an aliquot of cells. As a control reaction, an aliquot of competent cells to which no foreign DNA was added was treated similarly in all subsequent steps.

All tubes were stored on ice for 30 minutes and then heat shocked at 42°C for 90 seconds before immediately returning to ice. After chilling for 1-2 minutes, 800µl of SOC medium was added to each tube and the tubes were incubated at 37°C and 150 rpm for 1 hour. To allow for blue/white colour screening of transformant colonies 10µl of IPTG (0.1M) and 100µl of X-Gal (2% w/v) was spread over the surface of SOB agar plates supplemented with ampicillin (100 µg/ml final concentration) using a sterile spreader. Plates were allowed to dry for 1-2 hours. Transformed cells in SOC medium were then spread in 200µl aliquots onto the previously prepared SOB plates and residual liquid was allowed to absorb before incubating inverted plates at 37 °C for 12-16 hours.

2.7.2 Plasmid DNA isolation

In order to isolate high yields of good quality plasmid DNA for fluorescent sequencing, the Qiagen "QIAprep Spin Miniprep" kit was used according to the specifications of the manufacturer. For less sensitive downstream applications, such as probe preparation the alkaline lysis method of Sambrook *et al.* (1989) was used. A single colony (2-3mm diameter) was picked from a freshly grown plate and used to inoculate 2ml of LB broth supplemented with ampicillin (50µg/ml final concentration). Cultures were grown overnight at 37°C and 150 rpm. After overnight incubation, 1.5ml of the culture was transferred to a microfuge tube and cells were pelleted by centrifugation at 12,000g for 30 seconds at 4°C. The supernatant was removed and pellets allowed to air dry. For all subsequent steps the tube was kept on ice. The bacterial pellet was re-suspended in 100µl Solution I by vigorous vortexing and 200µl of freshly prepared Solution II was added in order to lyse the cells. Contents were mixed by rapid inversion of the tube but were not vortexed. DNA was then solublised in high salt by the addition of 150µl of Solution III and the tube briefly and gently vortexed in an inverted position. The tube was stored on ice for 3-5 minutes and high molecular weight chromosomal DNA and proteins were pelleted by centrifugation at 12,000g for 5 minutes at 4°C. The supernatant was transferred to a fresh tube and extracted once with an equal volume of

phenol:chloroform (1:1). Plasmid DNA was precipitated by the addition of 2 volumes of ethanol and the tube allowed to sit at room temperature for 5 minutes. After pelleting of plasmid DNA by centrifugation at 12,000g for 5 minutes at 4°C, the pellet was washed with 70% ethanol at 4 °C and was dried by brief vacuum drying. The pellet was then re-suspended in 50µl of sterile MilliQ water containing DNase free pancreatic RNase to a final concentration of 20µg/ml and stored at -20 ° C.

2.7.3 Electrophoresis of DNA on agarose gels

2.7.3.1 Agarose gel electrophoresis

Agarose gels were prepared in 1X TAE buffer containing ethidium bromide at a final concentration of 0.5µg/ml. Agarose was dissolved in 1X TAE and melted by several 30-second pulses in a microwave. Ethidium bromide was added after the molten gel had been allowed to cool to < 60 °C. Gel slabs were poured to a thickness of 0.5-1 cm with a gel comb in place to give sample wells of appropriate dimensions. Running buffer used was 1X TAE. DNA samples were prepared by dilution, if necessary, with MilliQ water and the addition of 6X gel loading buffer (Type IV) to a final concentration of 1X per sample. Either lambda (λ) DNA digested with *Hin*dIII, 1kb, or 100bp DNA ladders (New England Biolabs) were used as a molecular weight marker. Electrophoresis was carried out at 4-8 V/cm for 1-3 hours. DNA bands were visualised and documented under UV light using a UVP white/UV transilluminator and digital graphics printer.
Different agarose concentrations were used depending on the expected DNA size in order to give the best resolution of DNA bands as shown in table 2.7a below.

Agarose gel concentration (%)	**Efficient range of separation of linear DNA (kb)**
0.3	60 - 5
0.5	25 – 1.5
0.7	10 – 0.8
1.0	7 – 0.5
1.2	6 – 0.4
1.5	3 – 0.2
2.0	2 – 0.1

Table 2.7 a Agarose gel electrophoresis. Efficient range of separation of linear DNA by various gel concentrations.

2.7.3.2 Quantification of DNA on agarose gels

For quantification of DNA, known volumes of the DNA sample were electrophoresed alongside marker DNA of known volumes and concentration. For small DNAs such as plasmid DNA, probe DNA or PCR products, the marker DNA used was lambda (λ) DNA digested with *Hin*dIII. Sample DNA concentration was then estimated by comparison of band intensity with a marker band of similar size as shown in table 2.7b. For quantification of large DNAs such as genomic DNA or phage DNA, dilutions of wild type lambda (λ) DNA of known concentration were used as the DNA standard.

Lambda *Hin*dIII digested DNA band size (kb)	**% Total DNA**
23.13	48
9.42	19
6.56	14
4.36	9
2.32	5
2.03	4
0.56	1

Table 2.7b Quantification of DNA in agarose gels by comparison with *Hin*dIII digested lambda (λ) DNA markers.

2.7.3.3 Recovery of DNA from agarose gels

PCR product or vector insert DNA bands of interest were recovered from agarose gels using either the Pharmacia Biotech "Sephaglass Band Prep" kit or the Qiagen "QIAEX II Gel Extraction " kit according to the instructions of the manufacturer.

2.7.4 Restriction digestion of plasmid and phage DNA

DNA solutions of known concentration were mixed with restriction enzymes in appropriate buffer according to the specifications of the manufacturer (Kramel Biotechnologies, New England Biolabs, Bioline or Advanced Biotechnologies), and reactions were incubated at 37°C for 1 to 2 hours. Care was taken to ensure that the volume of restriction enzyme did not exceed 1/10 the final restriction volume, in order

to avoid inhibition of enzyme activity by glycerol in which commercial enzyme preparations are stored. The reaction was stopped by transferring the tubes to an ice bath or, where necessary, by the addition of 0.5M EDTA (pH 8.0) to a final volume of 20mM.

2.7.5 Ligation reactions

Digested vector and insert DNAs were mixed in a microfuge tube in molar ratios of 1:1 and 1:3 (vector:insert) calculated using the formula shown below:

$$\frac{\text{ng of vector x kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert required}$$

Ligase buffer containing ATP was added to a final concentration of 1X and 4 Weiss units of T4 DNA ligase (Promega) were added. For cohesive end ligations the ligation reactions were stored at RT for 3 hours and incubated at 4 °C overnight.

2.7.6 cDNA library screening

2.7.6.1 Preparation of plating cells

10ml of M broth was inoculated with a single colony of freshly grown *E.coli* NM514 and incubated overnight on a shaking incubator at 37°C. 1ml of the overnight culture was added to 50ml of pre-warmed M broth and incubated at 37°C with shaking until the cells had grown to an OD_{600} = 0.5 (~2.5 x 10^8 cells/ml, approximately 3 hours). The culture was cooled on ice, transferred to a polypropylene tube and centrifuged at 3000 rpm for 10 minutes on a Centaur MSE bench centrifuge. The supernatant was discarded and the pellet re-suspended in 15ml of filter sterile 10mM ice cold $MgSO_4$ and stored at 4°C.

2.7.6.2 Infection of plating cells with phage

Lambda phage particles were diluted in SM buffer to give the desired number of pfu/ml. For small (90mm) LB agar plates 100µl of plating cells and 100µl of diluted lambda were gently mixed in a sterilin tube, and incubated at 37° C for 15 - 20 minutes to allow the phage particles to adsorb to the cells. For large (140 mm) plates 250 µl of plating cells and diluted lambda were used. 4 - 8 ml of molten top agar/agarose (~ 45 °C) was added to each tube and gently mixed before pouring on to a pre-warmed LB agar plate. The plates were allowed to set for at least 15 minutes and incubated inverted at 37° C for 6 - 16 hours.

2.7.6.3 Preparation of replica filters for screening of λ gt10 cDNA library

Plates for screening were stored at 4 °C for at least one hour. Nylon membrane (Hybond N+) was placed over the surface for 1 minute. The membrane was transferred with a forceps and placed plaque side up for 7 minutes on a piece of Whatman 3MM paper soaked in denaturing solution. The membrane was then transferred to Whatman 3MM paper soaked in neutralising solution for 3 minutes. This step was repeated once, and the membrane was rinsed briefly in 2X SSC and allowed to air dry for one hour. DNA fixation was carried out by the alkali transfer method (2.7.6.6). Membranes were used immediately or wrapped in saran wrap and stored at -20 °C

2.7.6.4 Hybridisation with heterologous radiolabelled probes (Method 1)

If required in order to reduce background for plaque lift filters, the filters were pre-washed for 30 - 60 minutes in pre-warmed 5X SSC, 0.5X SDS at 60 °C. Membranes were placed in a plastic lunch box and immersed in a sufficient volume of pre-hybridisation solution to just cover the membrane. Fragmented herring sperm DNA (Sigma) was denatured by heating to 95 –100 °C for 5 - 10 minutes, cooling on ice for 2 minutes and added to pre-hybridisation solution at a final concentration of 100μg/ml

Prehybridisation was continued at 60 °C for at least 1 hour. For preparation of radiolabelled probe 10 - 50 ng of DNA was labelled with α ^{32}P dCTP using the "Ready to Go dCTP labelling kit" (Pharmacia) or the Amersham Pharmacia Biotech "Oligolabelling kit" according to the specifications of the manufacturer. Probe was denatured by heating to 100 °C for 10 minutes and snap cooling on ice for 2 minutes. Labelled probe was then added to the pre-hybridisation solution and hybridisation was allowed to proceed overnight at the required temperature.

Blots were then washed to appropriate stringency conditions. For heterologous probe applications usual stringency conditions were washing twice in 2X SSC, 0.1% SDS at room temperature and finally washed with 1x SSC, 0.1 % SDS at 60 °C for 30 minutes. Blots were then removed, excess liquid drained off, wrapped in saran wrap and placed with Kodak autoradiographic film in an autoradiography cassette with intensifying screens at - 70 °C. Autoradiographs were developed using an X-omat 2X processor.

2.7.6.5 Hybridisation with radiolabelled probes (Method 2)

Membranes from plaque lifts were placed in a plastic lunch box and covered with sufficient volume of pre-hybridisation solution to completely cover the membranes.

Heterologous fragmented DNA was not required as a blocking agent. Membranes were pre-hybridised at 60 °C for at least 1 h. For preparation of radiolabelled probe 20 - 50 ng of DNA was labelled with α ^{32}P dCTP using the Pharmacia Biotech "Oligolabelling kit" according to the specifications of the manufacturer. Labelled probe was denatured by heating to 100 °C for 5 minutes and snap cooling on ice for 2 minutes. Unincorporated nucleotides were removed by passing through a Sephadex G-50 column as described below (2.7.6.7). Prehybridisation solution was poured off and replaced with a small volume of fresh hybridisation solution. Labelled probe was then added to the hybridisation solution and hybridisation was allowed to proceed overnight at the required temperature.

Blots were washed twice for 30 minutes in low stringency (125mM sodium phosphate) wash solution at 60 °C; and once for 30 minutes in medium stringency (63mM sodium phosphate) wash solution at 60 °C. Where homologous probes were used the membranes were washed for a further 30 minutes in high stringency (15mM sodium phosphate) wash solution at 60 °C. (Low, medium and high stringency wash solutions contain 250mM, 125mM and 30mM and sodium ions respectively, and are equivalent to 1.7X, 0.8X and 0.2X SSC respectively). Membranes were then removed, excess liquid blotted off, wrapped in saran wrap and placed in an autoradiography cassette with intensifying screens. The membranes were covered with a sheet of X-ray film and autoradiograph cassettes stored at - 70 °C. Autoradiographic films were developed using an X-omat 2X processor.

2.7.6.6 DNA fixation to nylon membranes by alkali transfer

Two sheets of Whatman 3mm paper were placed in a plastic container and soaked in 0.4N NaOH. Dry membranes were placed DNA side up on the Whatman paper for 20 minutes and then briefly rinsed in 5X SSC to remove excess NaOH. Membranes were immediately used for hybridisation, or were wrapped in Saran wrap and stored at –20°C until required.

2.7.6.7 Removal of unincorporated nucleotides

Unincorporated radioactive nucleotides from labelling reactions were removed by passing through a freshly prepared Sephadex G-50 column. Columns were prepared by plugging the end of a 1ml syringe with sterile siliconised glass wool. Sephadex G-50 stock solution was added and packed down with the plunger to a column volume of 0.6 – 0.7ml and the column was equilibrated by passing two 200µl aliquots of column

buffer through the column. After probe labelling, the labelled probe solution was made up to 250µl with column buffer and passed through the column before denaturing.

2.7.7 PCR amplification of cDNA inserts in λ gt10

2.7.7.1 Template pre-amplification (mini liquid lysate)

10µl of NM514 plating cells and 10µl of cored out plaque eluate in SM buffer from second round screening were mixed in a microfuge tube and incubate at 37 °C for 25 minutes to allow phage to adsorb to the *E.coli* cells. 80µl of LB broth was added and tubes were incubated overnight at 37°C. Cell debris was removed by brief centrifugation at 13,000 rpm in a bench top centrifuge to pellet cells and the supernatant was used as the PCR template.

2.7.7.2 PCR amplification

For each reaction 1µl of template and 9µl of PCR reaction master mix were dispensed in a PCR tube and overlaid with a drop of sterile mineral oil. As a negative control a reaction containing no template DNA was included. As a positive control DNA template that had been previously successfully amplified was used. All tubes were placed in an MJ PTC100 thermocycler and PCR carried out using the following cycle conditions:

Step 1:	94 °C	3 min.
Step 2	94 °C	1 min
Step 3	52 °C	1 min
Step 4	72 °C	3 min
Step 5		go to 2 35 times
Step 6	72 °C	10 min
Step 7	4 °C	48h

After amplification, PCR products were analysed by gel electrophoresis.

2.7.8 Large Scale phage λ DNA extraction

2.7.8.1 Preparation of high titre phage lysate (> 2 x 10^{10} pfu/ml) - plate lysate method

Required plaques were cored out into 0.5ml of SM buffer in a screw capped Apex tube and a drop of high quality (e.g. HPLC grade) chloroform added. Samples were shaken briefly and stored at 4 °C for at least 2 h. Aliquots of 20, 10, 5 and 1µl were adsorbed to

50µl of fresh plating cells and plated out as previously described. After incubation at 37 °C overnight the best lysates were selected and 4ml of SM buffer was added to each plate. All plates were placed on a shaking incubator tray and shaken gently at 4 °C for 2-3 h. The phage eluate in SM buffer was poured into a centrifuge tube. The plate was rinsed with 1ml of SM buffer and the rinse was added to the tube. 4 -5 drops of chloroform (HPLC grade) were added, the tube vortexed briefly and centrifuged at 4000rpm for 10 minutes. The supernatant was transferred to a fresh tube and stored at 4°C.

2.7.8.2 Phage DNA amplification and isolation

A modification of the infection at high multiplicity protocol of Sambrook *et al.* (1989) was used. 10ml NYZCM medium was inoculated with 10µl of NM514 plating cells in a 100ml flask and incubated at 37°C and 120rpm overnight. The following morning 200ml of pre-warmed NYZCM was inoculated with 1ml of the overnight culture and incubated for ~3h until the $OD_{600} = 5$. 1ml of plate lysate was added to the culture and incubation was continued for 4-5 h at 37 °C and 150rpm until complete lysis of the bacterial culture occurred (visible as a fine splintery precipitate and/or large stringy clumps of bacterial debris). The culture was transferred in 2x 100ml aliquots to sterile centrifuge tubes and centrifuged at 10,000rpm at 4°C for 15 minutes in a Sorvall centrifuge. The supernatant was transferred to a clean centrifuge tube and 10µl RNase (10mg/ml) and 10µl of DNase I (10mg/ml) were added to a final concentration of 1mg /ml. All tubes were incubated at 37 °C for 1h. After incubation 5.8g NaCl was added to each sample (final concentration 1M) and dissolved by swirling. Tubes were stored on ice for 1 hour to allow dissociation of phage from the bacterial debris. Tubes were centrifuged at 10,000rpm for 15 min at 4°C in a Sorvall GSA rotor. The supernatant was transferred and 10g of PEG 8000 added to a final concentration of 10% in order to precipitate phage particles. Tubes were stored on ice for at least 1 hour. After centrifugation at 10,000rpm for 15 min at 4°C the supernatant was discarded and remaining fluid removed. The pellet was re-suspended in 2ml of SM buffer, rinsing the walls of the centrifuge tube thoroughly to remove phage precipitate. 2ml of chloroform (HPLC grade) was added and tubes vortexed gently for 30s. After centrifugation at 3000rpm for 15 min at 4°C in a Sorvall centrifuge the aqueous phase containing phage particles was recovered and transferred to FEP Sorvall centrifuge tubes suitable for use with an SS34 rotor.

2.7.8.3 Extraction of phage DNA

The following reagents were added to the supernatant - 0.5M EDTA (pH 8) to a final concentration of 20mM, Proteinase K (20mg/ml) to a final concentration of 50μg/ml, and 10% SDS to a final concentration of 0.5%. Components were mixed by inversion and incubated at 56°C for 1 hour. An equal volume of equilibrated phenol (pH 7.9) was then added and tubes were centrifuged at 3000rpm for 10 minutes at 4°C in an SS34 rotor. The aqueous phase was dispensed in 750μl aliquots into microfuge tubes and extracted twice with phenol: chloroform: isoamyl alcohol 25: 24: 1. The upper aqueous phase was then extracted once with chloroform and centrifuged at 3000rpm for 10 minutes at 4°C in a bench-top centrifuge. Phage DNA was precipitated with 0.1 volumes of 3M sodium acetate (pH 6) and 2.5 volumes of 95% ethanol and stored at -70°C for 30 minutes. After centrifugation at 14,000rpm for 30 minutes the supernatant was discarded and the pellet washed with 1ml of 70% ethanol. The pellet was allowed to air dry and re-suspended in 70μl of sterile MilliQ water.

2.7.9 Genomic DNA isolation and Southern blotting

2.7.9.1 Cassava genomic DNA extraction

The method of Dellaporta *et al.* (1983) was used. Young leaf tissue (cultivar MNGA 1) was collected and ground to a fine powder in liquid nitrogen. 4g of ground tissue was transferred to a centrifuge tube and 15ml of extraction buffer (pre-warmed at 50 °C), and 1ml of 20% SDS was added. The tubes were mixed well and incubated in a water bath at 65°C for 10 minutes. Tubes were mixed by inversion every 2 minutes. Tubes were removed and 5ml of ice-cold 5M potassium acetate was added with vigorous mixing. Tubes were placed on ice for 1 hour and centrifuged at 10,000rpm for 20 minutes at 4°C in a Sorvall SS34 rotor. The supernatant was filtered through sterile muslin to a fresh tube containing 10ml of isopropanol pre-cooled at -20 °C. The tubes were gently mixed by inversion and stored at -20 °C for at least 2 hours or overnight. After centrifugation at 10,000rpm for 15 minutes at 4°C the supernatant was discarded and the pellet washed with 70% ethanol. The pellet was then allowed to air dry and redissolved in 700μl of sterile MilliQ water. The solution was transferred to a microfuge tube and DNase free RNase was added to a final concentration of 10μg/ml and incubated at 37 °C for 30 minutes. The DNA solution was extracted once with phenol:

chloroform: isoamyl alcohol 25: 24: 1. The aqueous phase was transferred, and the DNA precipitated by the addition of 75µl of 3M sodium acetate (pH 5.2) and 500µl of isopropanol (pre-cooled to -20°C). The solution was mixed gently by inverting the tubes and all tubes were placed at -20°C for at least 2 hours or overnight. Tubes were then centrifuged at 15,000rpm for 15 minutes at 4°C and the supernatant discarded. Each pellet was washed twice with 500µl of 70% ethanol and was allowed to air dry for at least 15 minutes. Pellets were then re-suspended in 100-200µl sterile MilliQ water and the integrity of the DNA was checked by electrophoresis on a 0.8% gel. DNA quantifications were carried out as described in section 2.7.3.2. If required the genomic DNA preparations were concentrated by ethanol precipitation and re-suspension in a smaller volume.

2.7.9. 2 Ethanol precipitation of DNA

0.1 volumes of 3M sodium acetate (or 0.25 volumes of 10M ammonium acetate) and 2.5 volumes of 95% ethanol were added to the DNA sample and tubes were placed on ice for 15-20 minutes. After centrifugation at 12,000rpm for 15 minutes in a bench-top centrifuge the supernatant was discarded and the pellet was re-suspended in 1ml of ice cold 70% ethanol. The tube was centrifuged as previously, the supernatant discarded and the pellet allowed to air dry for at least 10 minutes before re-suspension in a suitable volume of sterile MilliQ water.

2.7.9.3 Restriction digestion of genomic DNA

Restriction digests were set up according to the specification of the manufacturer as below:

Reaction component:	final concentration:
DNA	10 - 20 µg
10x buffer	1x
enzyme	72U
spermidine	4mM

Reactions were adjusted to a suitable volume with sterile MilliQ water. Care was taken to add spermidine last and to carefully stir the reactions after addition of each component as this helped to reduce "clumping" of the DNA. Restriction reactions were incubated at 37°C for 24-48 hours and the restriction enzyme was then inhibited by

addition of EDTA to a final concentration of 20 mM or by chilling on ice.

2.7.9.4 Southern blot preparation

Genomic DNA restriction digest samples were electrophoresed on a 0.8% TAE gel at 1 -2 v/cm overnight. The gel was then placed in depurinating solution and gently shaken in a plastic container for 15 minutes. The solution was poured off, the gel rinsed in distilled water and immersed in denaturing solution with shaking for 30 minutes. The gel was rinsed as previously and immersed in neutralising solution with shaking for 30 minutes. Southern blot apparatus was then set up. This consisted of a glass tray containing 5-10mm depth of 10X SSC. A plastic gel tray was placed inverted in the liquid and 2 sheets of Whatman 3mm paper soaked in 10X SSC were placed over the gel tray such that the ends were immersed in liquid to form a wick. Any air bubbles were gently removed by rolling with a clean plastic 100ml pipette. The gel was then placed inverted on the wet paper and overlaid with a piece of nylon membrane (Hybond N+) the same size as the gel, taking care to avoid air bubbles. 2 pieces of Whatman filter paper soaked in 2X SSC, of the same size as the gel, were placed above the membrane and Saran wrap was placed over the gel extending to the sides of the glass tray. The region of Saran wrap overlying the gel was cut away using a scalpel and a wad of absorbent tissue placed above the gel and filter paper. A glass plate was placed on top and a glass bottle (~ 500 g) added on top. After overnight transfer the nylon membrane was rinsed in 2X SSC, allowed to air dry for 1 hour and fixed by alkali transfer as previously described (section 2.7.6.6).

2.22.2 Southern hybridisation

Membranes for Southern blotting were placed in a glass hybridisation tube and covered with a sufficient volume of Southern pre-hybridisation solution to cover the membrane. Heterologous fragmented DNA was not required as a blocking agent. Membranes were pre-hybridised at 60°C for at least 1 hour. For preparation of radiolabelled probe 20-50ng of DNA was labelled with α^{32}P dCTP using the Pharmacia Biotech "Oligolabelling kit" according to the specifications of the manufacturer. Labelled probe was denatured by heating to 100°C for 5 minutes and snap cooling on ice for 2 minutes. Unincorporated nucleotides were removed by passing through a Sephadex G-50 column as described previously (section 2.7.6.7). Prehybridisation solution was poured off and replaced with a small volume of fresh hybridisation solution. Labelled probe was then added to the hybridisation solution and hybridisation was allowed to proceed overnight.

Following hybridisation, membranes were washed briefly (5-10 minutes) in low stringency wash solution at room temperature and twice for 20 minutes in low stringency wash solution at 60°C in order to allow detection of related gene family members. For high stringency washing to detect the cognate gene only, membranes were washed for two further 30 minute washes in high stringency wash solution at 60°C. (Low and high stringency wash solution salt concentrations were calculated for each experiment taking into account the expected level of homology using the formula $Tm°C = 81.5°C + 16.6 \log [Na^+] + 0.41(\%GC) - (600/l)$ as shown in table 2.7.9a for a sample probe of size 700bp. After appropriate washing, membranes were removed, excess liquid blotted off, wrapped in Saran wrap and placed with autoradiographic film (Kodak) in an autoradiography cassette with intensifying screens at -70°C. Autoradiographs were developed using an X-omat 2X processor.

Salinity (SSC concentration)

T°C	10X	6X	4X	2X	1X	0.5X	0.2X	0.1X	0.05X	0.01X
40	57.3	59.8	61.7	65.1	68.4	71.7	76.1	79.5	82.8	90.5
45	60.7	63.1	65.1	68.4	71.7	75.0	79.5	82.8	86.1	93.9
50	64.0	66.4	68.4	71.7	75.1	78.4	82.8	86.1	89.4	97.2
55	67.3	69.8	71.7	75.1	78.4	81.7	86.1	89.5	92.8	
60	70.7	73.1	75.1	78.4	81.7	85.0	89.5	92.8	96.1	
65	74.0	76.4	78.4	81.7	85.1	88.4	92.8	96.1	99.4	
68	76.0	78.4	80.4	83.7	87.1	90.4	94.8	98.1		
70	77.3	79.8	81.7	85.1	88.4	91.7	96.1	99.5		

Table 2.7.9a. Percentage of DNA:DNA homology required to form a stable hybrid as a function of temperature and salinity. The values above were calculated using the formula:
$Tm°C = 81.5°C + 16.6 \log [Na^+] + 0.41(\%GC) - (600/l)$
where l is the length of probe used.
The table above shows a worked example for a probe of length 700bp and assuming a GC content of 50%. A salt concentration of 1X SSC = 0.15M Na^+, and it is assumed that a change of 1.5°C is equivalent to a change of 1% homology.

2.7.10 RNA isolation and northern blotting

2.7.10.1 Cassava root RNA extraction

Root tissue for RNA extraction was grated using a domestic cheese grater in order to facilitate subsequent grinding, wrapped in aluminium foil and immediately frozen in liquid nitrogen. Where the sample was not processed immediately, it was stored at -70 °C until required. For processing, grated tissue was homogenised to a fine powder using

a porcelain pestle and mortar previously chilled to -70°C. Aliquots of liquid nitrogen were continuously added during grinding to prevent thawing and consequent degradation by RNases in the sample. 3g of powdered material were transferred to a 15ml plastic tube that had been chilled by immersion in liquid nitrogen. 15ml of extraction buffer (pre-warmed at 60°C) and 15ml of chloroform: isoamyl alcohol (24: 1) added and the tube vortexed vigorously. The mixture was dispensed in 2ml aliquots into screw capped tubes and centrifuged at 12,000rpm in a bench centrifuge for 5 minutes. The upper phase was transferred and the chloroform extraction repeated twice. Lithium chloride (8M) was added to 0.33 volumes and the tubes incubated at 4 °C overnight. Tubes were centrifuged at 12,000rpm for 5 minutes, the supernatant discarded and the pellet re-dissolved in DMPC or DEPC treated MilliQ water. After a chloroform extraction step and a phenol: chloroform: isoamyl alcohol (25: 24: 1) extraction step, the RNA was precipitated with 0.25 volumes of 5M NaCl and 2 volumes of ethanol for 30 minutes at -70°C. Tubes were then centrifuged at 12,000rpm for 20 minutes. The pellet was washed with 70% ethanol, dried for 1-5 minutes in a speed vac concentrator and dissolved in 50µl DMPC treated water or formamide. To assess the quality and quantity of RNA, aliquots were run on a 2% TAE gel and were used for spectrophotometer readings at A_{260} and A_{280}.

2.7.10.2 Cassava leaf RNA extraction

Leaf samples for RNA extraction were harvested, wrapped in aluminium foil and immediately frozen in liquid nitrogen. Where the sample was not processed immediately, it was stored at -70 °C until required. Tissue was homogenised to a fine powder using a porcelain pestle and mortar previously chilled to -70 °C. Aliquots of liquid nitrogen were continuously added during grinding to prevent thawing and consequent degradation by endogenous RNases. A 2ml screw cap tube was half filled with homogenised tissue using a pre-chilled spatula and 1ml of extraction buffer added. The tube was vortexed vigorously and an equal volume of phenol added. After vortexing the tube was centrifuged at 12,000g for 5 minutes at 4 °C in a bench-top centrifuge. The aqueous phase was transferred and extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1), and once with an equal volume of chloroform. RNA was precipitated by addition of 1 volume of pre-chilled isopropanol at -70°C for 15 minutes. The tube was centrifuged as previously, the supernant was discarded and the pellet re-suspended in 500µl DEPC or DMPC treated water and the isopropanol precipitation step repeated once more. The pellet was then dissolved in

500µl DEPC or DMPC treated water, 0.33 volumes of 8M LiCl were added and RNA allowed to precipitate overnight at 4°C. After overnight precipitation the tube was centrifuged at 12,000g for 5 minutes and the pellet re-suspended in 300µl DEPC or DMPC treated water. 0.1 volumes of 3M sodium acetate (pH 4.8) and 2 volumes of 70% ethanol were added and the RNA precipitated at -70 °C for 30 minutes. After centrifugation at 12,000g for 10 minutes at 4 °C the supernatant was discarded, the pellet washed was with 70% ethanol, dried in a speed vac concentrator for 5 minutes and dissolved in 50-100µl of formamide or DEPC treated water. To assess the quality and quantity of RNA, aliquots were run on a 2% TAE gel and used for spectrophotometer readings.

2.7.10.3 Electrophoresis of RNA on denaturing formaldehyde gels

Prior to electrophoresis the gel apparatus, gel combs and gel tray were thoroughly cleaned with water, wiped carefully with RNaseZap (Ambion), and thoroughly rinsed with DEPC treated water. A denaturing formaldehyde gel was prepared in a chemical hood and allowed to set for at least 30 minutes. RNA samples were made up to a final volume of either 20µl or 10µl as below:

		Final concentration
RNA samples in formamide:	10µl (5 µl)	5-10 µg
DEPC treated MilliQ	4.5µl (2.25µl)	----
Formaldehyde	3.5 µl (1.75 µl)	2.2M
5X MOPS buffer	2 µl (1µl)	1X

Marker RNA (NEB) was prepared as below for different gel well sizes. For small wells (10µl capacity) 3µl of RNA ladder (New England Biolabs) was combined with 2µl 5X MOPS buffer, 1µl formaldehyde and made up to 10µl with RNase free water. For large sample wells, 3µl of RNA ladder was combined with 4µl 5X MOPS buffer, 2µl formaldehyde and made up to 20µl with RNase free water. All samples were incubated at 70°C for 5 minutes, chilled on ice and briefly centrifuged. Either 2 or 4 µl (for sample volumes of 10 or 20µl respectively) of sterile, DEPC treated, formaldehyde gel loading buffer (containing ethidium bromide to a final concentration of 0.05µg/ µl) was added to the RNA samples. Prior to loading of the samples the gel was pre-run at 5V/cm for 5 minutes. Samples were loaded with marker RNA on an outside lane, and the gel was run submerged in 1X MOPS buffer at 3-4 V/cm until the bromophenol blue had migrated 4-6cm.

2.7.10.4 Northern blotting

After electrophoresis, the gel was soaked in several rinses of DEPC MilliQ water to remove excess formaldehyde. If required in order to assess equal loading the gel was briefly examined and photographed under UV light. Whilst the gel was soaking the Northern blot apparatus was set up. This consisted of a clean RNase free plastic tray filled with 20X SSC. A plastic gel tray was placed inverted in the liquid and 2 sheets of Whatman 3mm paper soaked in 20X SSC were placed over the gel tray such that the ends were immersed in liquid to form a wick. Any air bubbles were gently removed by rolling with a freshly opened sterile plastic 100ml pipette. The gel was then placed inverted on the wet paper and overlaid with a piece of nylon membrane the same size as the gel, taking care to avoid air bubbles. 2 pieces of Whatman filter paper soaked in 2X SSC, of the same size as the gel, were placed above the membrane and Saran wrap was placed over the gel extending to the sides of the glass tray to prevent evaporation and "short circuiting". The region of Saran wrap overlying the gel was cut away using a scalpel and a wad of absorbent tissue placed above the gel and filter paper. A glass plate was placed on top and a glass bottle (~ 500 g) added on top. After overnight transfer the nylon membrane was marked with pencil to indicate lane orientation. The damp membrane was placed RNA side down on a clean RNase free UV transilluminator for 3 minutes in order to fix the RNA to the membrane and then briefly rinsed in 2X SSC. Membranes were either used immediately for hybridisation or were placed between 2 sheets of Whatman 3MM paper, wrapped in aluminium foil and stored at room temperature.

2.7.10.5 Staining of marker RNAs

After transfer and fixing of RNA to Nylon membranes, the area of membrane containing the marker lane was carefully cut out and immersed in methylene blue staining solution overnight. Before documenting the membrane was briefly de-stained by rinsing in RNase free water.

2.7.10.6 Northern hybridisation

Membranes for Northern blotting were placed in a glass hybridisation tube and covered with a sufficient volume of pre-hybridisation solution. Membranes were pre-hybridised at 65°C for at least 1 hour. For preparation of radiolabelled probe 20-50ng of DNA was labelled with α^{32}P dCTP using the Pharmacia Biotech "Oligolabelling kit" according to

the specifications of the manufacturer. Labelled probe was denatured by heating to 100 °C for 5 minutes and snap cooling on ice for 2 minutes. Unincorporated nucleotides were removed by passing through a Sephadex G-50 column as described previously (section 2.7.6.7). Prehybridisation solution was poured off and replaced with a small volume of fresh hybridisation solution. Labelled probe was then added to the hybridisation solution and hybridisation was allowed to proceed overnight at 65°C.
Following hybridisation, membranes were washed twice for 1-2 minutes in 1X SSC, 0.1% SDS followed by 2-3 washes of 15 minutes in 0.2X SSC, 0.2% SDS 65°C. Membranes were then removed, excess liquid blotted off, wrapped in Saran wrap and placed in an autoradiography cassette with intensifying screens. The membranes were covered with a sheet of autoradiographic film, placed in an autoradiograph cassette with intensifying screens and stored at -70°C. Autoradiographic films were developed using an X-omat 2X processor. In order to ensure a linear response, autoradiograph films for northern blots were pre-flashed for less than a millisecond using a calibrated stroboscope.

2.7.10.7 Stroboscope calibration for pre-flashing of autoradiograph film

Photographic emulsions on autoradiographic film are disproportionately insensitive to low light intensities. Thus, when intensifying screens are used, the response of image formation is non linear, as areas of lower photon emission are under represented. In order to allow comparison of band intensities in the final autoradiograph, the films are pre-flashed to ensure a linear response. To calibrate the stroboscope, the lamp was covered with a yellow filter and suspended from an adjustable tripod set up above a flat bench surface. Working in complete darkness, the height of the lamp above the bench surface was adjusted and at each height a piece of autoradiographic film was exposed to a single flash of light. The pieces of film were developed as was a piece of non pre-flashed film. The absorbance at 545nm was measured in a spectrophotometer using non pre-flashed film as a "blank" and the distance that caused an increase in absorbance of 0.15 in the film was selected.

2.7.10.8 Root treatments used for northern blot experiments

Pre-harvest pruning treatment: roots were obtained from plants which had been pruned by removal of the stem at a height of approximately 30cm from the ground 2 weeks prior to harvest

Ethylene treatment: root slices were incubated in the ethylene generating compound

ethephon (Sigma) (0.02% in sterile water) for 24 hours in the dark. Control slices were incubated in water alone.

Methyl jasmonate treatment: root slices were incubated for 24 hours in the dark in methyl jasmonate (Sigma) (500μM in 0.1% ethanol).

For both the ethylene and methyl jasmonate treatments, samples of transverse root slices (approximately 1.5cm thickness) for control and experimental treatments were placed in a Petri dish and immersed to a depth of approximately 0.5cm in the appropriate treatment solution. The dish lids were loosely replaced to prevent excessive escape of gas in the case of the ethephon treatment, whilst still allowing air access.

2.7.11 *In vivo* detection and localisation of reactive oxygen species

2.7.11.1 Detection of superoxide by vacuum infiltration with NBT

Freshly harvested roots were immediately cut into a sufficient number of tissue slices for the experiment. At each time point, slices were vacuum infiltrated with 3ml of 0.05% NBT (Nitroblue tetrazolium) in 10mM K_2HPO_4 (pH 6) and incubated at RT for 15 minutes according to a modification of the method of May *et al.* (1996) (Vallelian-Bindschedller pers. com.). After incubation chloral hydrate (4mg/ml) was added to stop the reaction and the tissue slices were documented by photography or by direct scanning of the tissue slices.

2.7.11.2 Histochemical stain for the detection and localisation of superoxide

For microscopic observation, thin hand cut root sections were prepared using a fresh razor blade and were stained for the presence of superoxide using the method of Ros Barcelo (1998). Sections were incubated directly in 0.25mM NBT (Nitroblue tetrazolium) in 50mM potassium phoshate buffer (pH 7.8) for 15-20 minutes. Sections were transferred to a glass slide and were examined by light microscopy.

2.7.11.3 Detection of hydrogen peroxide by vacuum infiltration with DAB

At each time point, hand cut root slices were vacuum infiltrated with 3ml of DAB (3,3 diaminobenzidine tetrahydrochloride) (2mg/ml) and incubated at RT for 3 hours according to the method of Thordal-Christensen *et al.* (1997). After incubation chloral hydrate (4mg/ml) was added to stop the reaction and the tissue slices were documented by photography or by direct scanning of the tissue slices. As a control, similar tissue slices at each time point were co-infiltrated with DAB (2mg/ml) and 10mM ascorbate

(scavenger of H_2O_2). For microscopic observation thin hand cut sections were prepared using a fresh razor blade and were vacuum infiltrated as above. Sections were placed on a glass slide and incubated for 3 hours as above, and were then examined by light microscopy.

2.7.12 *In Vitro* quantification of reactive oxygen species

2.7.12.1 Quantification of hydrogen peroxide in cassava roots

A modification of the method of Warm and Laties (1982) was used. Root tissue (0.05g) was thoroughly ground in 1ml of 5% metaphosphoric acid. Root extract and washings were transferred to a microfuge tube and centrifuged at 10,000rpm for 10 minutes. For each sample to be processed, 2 Dowex basic anion exchange resin batch columns were prepared just prior to use. Each column was prepared by pipetting 1ml of Dowex slurry in deionised water into a 1.5ml microfuge tube. Tubes were centrifuged at 10,000rpm for 1 minute to give a packed bed volume of 0.5ml. The deionised water supernatant was removed and replaced with 1ml of 5% metaphosphoric acid; the slurry was re-suspended by brief vortexing and centrifuged as previously. The supernatant was again discarded and 0.5ml of the root extract supernatant was added to the resin. The slurry was re-suspended by brief vortexing and incubated on a shaker table at 150rpm for 5 minutes. The batch column was centrifuged at 10,000rpm for 1 minute. The supernatant was removed and passed through the second Dowex batch column as previously. For the measurement of hydrogen peroxide, a micro titre plate was loaded with triplicate samples of plant extract and blank reactions made up as below:

Plant extract reactions:	
Tris.HCl buffer (pH 8.5)	195µl
Luminol	25 µl
Root extract	5 µl
Blank reactions:	
Tris.HCl buffer (pH 8.5)	200µl
Luminol	25 µl

The micro titre plate was placed in a luminometer and luminescence measured and recorded using the Winglow software programme. For each sample, luminescence was initiated by automated injection of 25µl of potassium ferricyanide, and emitted photons were measured over a time period of 15 seconds after delay of 5 seconds.

For the standard curve aliquots of 10mM hydrogen peroxide, freshly diluted in 5% metaphosphoric acid, were made up to 200µl with 0.2M Tris.HCl buffer (pH 8.5) in the wells of a micro titre plate. 25µl of luminol was added, the plate was placed in the luminometer and luminescence was initiated by automated injection of 25µl of potassium ferricyanide and measured as previously. The standard curve is shown below (figure 2.7.12.1).

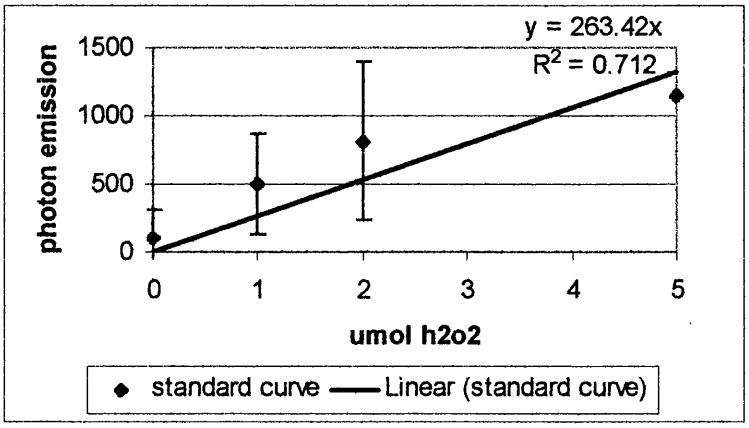


Figure 2.7.12.1 Standard curve for quantification of hydrogen peroxide

2.7.13 Detection of non-enzymatic antioxidants by thin layer chromatography

2.7.13.1 Root extract sample preparation

Root samples for analysis were ground in liquid nitrogen to a fine powder. 1g of powdered tissue were suspended in 10ml of HPLC grade ethanol in a 50ml tube, and blended using an Ultra Turrex blender. Samples were stored at RT for 30 minutes and were then filtered through Whatman number 1 filter paper. Samples and washings were transferred to a vaccuum flask and evaporated until completely dry. Each sample was re-suspended in 1ml HPLC grade ethanol and dissolved by sonication for 30-60 seconds. Samples were then filtered through a nylon filter (HPLC technology, pore size 0.22µm), the filtrate transferred to brown glass vials and stored at 4°C until required.

2.7.13.2 TLC separation using silica gels

A base line was pencilled 3cm from the base of the plate (HPTLC silica gel 60 F_{254} (Merck)) and divided into 1cm sample lanes divided by 0.5cm intervals using a soft lead pencil to avoid scratching of the plate. For each sample, 100µl was spotted along the 1cm lane in 20µl aliquots. Spotting was carried out working close to a hairdryer on a stand and sample aliquots were allowed to dry completely before addition of subsequent aliquots. Once the silica plate had been loaded it was briefly placed at 100°C to ensure

all samples were completely dried. The silica plate was immersed to a depth of 2cm in solvent mixture chloroform: ethyl acetate: methanol (2: 2: 1)) and the solvent front was allowed to migrate approximately half the height of the silica plate. The silica plate was then examined and documented under UV light (254nm and 366nm) and observed bands circled using a soft pencil. For the detection of antioxidants the plate was carefully sprayed with a fine mist of DPPH (1,1 Diphenyl-2-picryl-hydrazl) staining reagent and documented by photography.

2.7.14 Tissue print and *in vivo* detection and localisation of enzyme activity

2.7.14.1 Tissue print detection and localisation of catalase activity

A modification of the starch gel method (method 2) of Manchenko (1994) was used. Starch paper was prepared by soaking pieces of nitrocellulose membrane (Hybond C Amersham) in 1% starch solution (1% soluble starch in MilliQ water - microwaved to dissolve and allowed to cool) for 1 minute. Membranes were placed on filter paper and gently blotted with tissue to remove excess drops of solution, and were allowed to dry completely. Root slices for printing were hand cut using a fresh blade for each section and printed onto starch paper using firm even pressure for 30 seconds. 15ml of solution A and 35ml of solution B were quickly mixed just before use, and the membrane placed in this solution for 60 seconds with gentle swirling. Membranes were transferred to 50ml of solution C for 60 seconds, placed on filter paper and documented by photography after 10 minutes.

2.7.14.2 Tissue print detection of peroxidase activity

A modification of the method of Peyrado *et al.* (1996) was used. Root slices were cut by hand using a fresh blade for each section. Nitrocellulose membrane was placed on a piece of Whatman paper and tissue prints were prepared by gently pressing the tissue section onto the nitrocellulose membrane for 30 seconds. The membrane was placed in a Petri dish on a piece of Whatman filter paper soaked in 50mM phosphate buffer pH 5.3. Aliquots of 250µl detection solution (0.1 % H_2O_2 in 10mM aqueous guiacol) were added above the print and colour was allowed to develop for 5 minutes before documenting by photography or by direct scanning of the prints.

For *in vivo* detection and localisation of peroxidase activity, thin hand cut sections were prepared using a fresh razor blade. Sections were placed on a glass slide and immersed in detection solution. After 5 minutes the sections were examined by light microscopy.

2.7.14. 3 India ink staining of immobilised total protein

A modification of the method of Sambrook *et al.* (1989). Tissue prints on nitrocellulose paper were allowed to air dry, then floated on distilled water to allow them to wet from below by capillary action. Prints were then submerged for ~5 minutes to displace air bubbles and transferred to 0.4% Tween 20 in phosphate buffered saline for 5 minutes. Prints were transferred to fresh solution for 5 minutes and then placed in a solution of India ink (500µl in 50ml of 0.4% Tween 20 in phosphate buffered saline) and incubated for ~ 1 hour until the stain reached the desired intensity. Prints were then rinsed in several changes of phosphate buffered saline until background staining was removed.

2.7.14.4 Coomassie blue staining of immobilised total protein

A modification of the method of Sambrook *et al.* (1989) as described by Mas and Pallas (1995) was used. Tissue prints on nitrocellulose membranes were placed in staining solution for 1 minute, rinsed in distilled water and placed in de-staining solution for several minutes. Membranes were placed on Whatman filter paper and allowed to dry before photographing.

2.7.14.5 Phloroglucinol staining of cassava root tissue

Tissue sections were covered with a 5% phloroglucinol solution in methanol for 4 minutes, drained and irrigated with 1:1 HCl and water to remove excess stain. Lignified and suberised tissues stained a deep red.

2.7.14.6 Toluidine blue staining of cassava root tissue

Tissue sections were covered were covered with toluidine blue staining reagent (1% in citrate phosphate buffer pH 4). Lignified tissues stained blue, unlignified cell walls stained red/purple.

2.7.15 Protein extraction and analysis

2.7.15.1 Protein extraction from cassava storage roots

At each time point root tissue was grated using a standard cheese grater, wrapped in aluminium foil and frozen in liquid nitrogen. Samples were stored at –70 °C before use. The frozen tissue was ground under liquid nitrogen to a fine powder using a pre-chilled pestle and mortar. 5g of tissue was then added to a prechilled 50ml tube and 20ml of

extraction buffer and 0.25g of insoluble PVP (Polyclar AT BDH) was added. Tubes were vortexed vigorously and were placed in a container of ice on a shaker table (with the orientation of the tubes parallel to the direction of agitation) for 1 hour. Tubes were centrifuged at 5000rpm at 4 °C for 20 minutes in a Sorvall centrifuge. The supernatant was filtered through Miracloth into a tube capable of centrifugation at 11,000rpm and centrifuged at 4 °C for 20 minutes in a Sorvall SS34 rotor. The supernatant was transferred to a fresh tube and stored at -20 °C.

2.7.15.2 Protein concentration and purification

Dialysis tubing (Medicell International, MWCO 12-14000 Da) was prepared by cutting appropriate lengths of tubing and placing in a 1l bottle containing 10mM EDTA (disodium salt) and 100mM $NaHCO_4$ solution prepared with MilliQ water. The bottle was placed in a water bath at 60 °C for 1 – 2 hours, the solution was drained of and the tubing rinsed in MilliQ water twice for 30 minutes. After preparation the tubing was stored in MilliQ water containing 1ml/l of chloroform at 4 °C until required. For each sample, the end of a piece of dialysis tubing was sealed with a plastic clip and the sample pipetted into the tubing. The top was then sealed with a plastic clip and the sample placed on a layer of PEG 35,000 in a plastic container and stored at 4 °C until the sample volume had reduced by at least half. After concentration to an appropriate volume, the dialysis tubing was gently rinsed with MilliQ water to remove excess PEG and the sample transferred by pipetting into a microfuge tube and stored on ice or at -20 °C.

2.7.15.3 Determination of protein concentration

The protein content of each sample was determined using a modified Bradford protocol (Gomez-Vasquez, pers. com.) according to Stoscheck (1990). A calibration curve was constructed using BSA (Bovine Serum Albumin) as a standard. A series of standard protein reactions was set up by pipetting appropriate volumes of reagents in 1.5ml microfuge tubes as below.

Protein (BSA 1mg/ml)	MilliQ water	1.5M NaOH	Coomassie Blue G Reagent
0	20	50	1000
2	18	50	1000
5	15	50	1000
8	12	50	1000
12	8	50	1000
16	4	50	1000
18	2	50	1000

Table 2.7.15.a Volumes (µl) of each component for preparation of a modified Bradford standard curve.

For each protein concentration 3 replicate tubes were prepared. The tubes were mixed thoroughly by inversion and for each tube 3x 250µl aliquots were transferred to a micro titre plate. The reactions were incubated at room temperature for 5 minutes and the plate was then read at 595nm using a Dynatech MR5000 microplate reader. A calibration curve of the log [A_{595}] against log [protein content] was calculated using Microsoft Excel software as below (figure 2.7.15b) For determination of the protein content of each sample, 3 replicates of 20µl of sample were mixed with 50µl of 1.5M NaOH and 1ml of Coomassie blue G reagent. Triplicate 250µl aliquots were transferred to a micro titre plate and the absorbance at 595nm determined as previously. The protein content was determined by the intercept with the standard protein curve and the value obtained was multiplied by 53.5 to take into account the 20/1070 dilution of the protein sample for the protein determination assay.

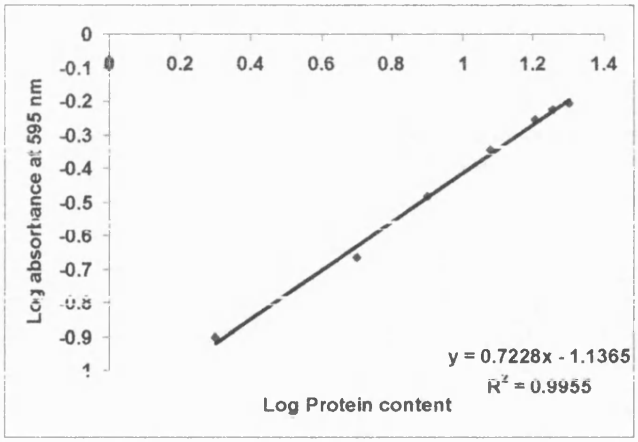


Figure 2.7.15b
Calibration curve for determination of protein content

2.7.15.4 Isoelectric focussing

An Ampholine PAG plate pH 3.5 – 9.5 (Amersham Pharmacia) was used and run on a

Multiphor II electrofocusing LKB BROMMA 2117 system at 16 °C. The machine was turned on to allow it to pre-cool about 20 minutes prior to loading the gel. The wells either side of the electrophoresis tank were filled with a small volume of freshly prepared 1M NaOH to absorb CO_2 which may affect the pH of the cathode and anode solutions during the run. A small volume (1 – 2ml) of liquid paraffin was applied to the electrophoresis plate and the screen print guide was smoothed onto the surface making sure no bubbles were trapped beneath the guide. An additional 1 – 2ml of liquid paraffin was placed on the guide print and the PAG plate carefully positioned above the guide, again ensuring no bubbles were present. The plastic cover of the plate was gently removed and electrode strips were prepared. For the cathode 3ml of 0.1M NaOH were pipetted onto the strip and allowed to soak in evenly. The strip was then placed on the gel with forceps. For the anode 0.04M aspartic acid was used. Sample application pieces were applied using forceps down the centre of the gel and a volume of protein extract corresponding to 30µg of protein was pipetted onto the sample application paper. 10µl of pI marker (Pharmacia Biotech) was run on one lane in order to allow approximation of the isoelectric point of protein isoforms. The gel was run at 1500V and 50mA with the power (W) setting at 30 for 45 minutes. The sample application papers were then removed and the gel run for a further 35 minutes. Care was taken to check the gel every 10 – 20 minutes and remove any condensation on the cover in order to avoid short circuits. After running, the pI marker was cut from the gel and stained with Coomassie blue as below. The remainder of the gel was then used for detection of protein isoforms by either direct staining of the gel or by gel overlay methods.

2.7.15.5 Coomassie blue staining of pI markers

The gel piece containing the marker was placed in a Petri dish and gently agitated in the solutions shown below for the appropriate amount of time.

Step	Time (minutes)	Solution
1	30 – 60	Fixing solution
2	5	MilliQ water
3	10	Staining solution
4	Overnight or until background is clear	De-staining solution
5	60	Preserving solution

Table 2.7.15c Staining procedure for pI markers (Parmacia Biotech)

2.7.15.6 Detection of peroxidase isoforms on IEF gels

After running the IEF gel was placed in a plastic container and immersed in 100ml detection solution (H_2O_2 0.1% v/v, aqueous guiacol 10mM, in 50mM phosphate buffer pH 5.2) with gentle agitation for 1 hour. Peroxidase bands stained a dark brown colour. The gel was documented by photography and the distance of isoform bands with reference to the cathode was measured.

2.7.15.7 Detection of superoxide dismutase by a protein overlay method

A modification of the method of Manchenko (1994) was used. A gel overlay (1.5% agarose in 50mM Tris.HCl pH 8.5) was prepared by heating the solution in a microwave until molten, and pipetting between 2 glass plates to form a gel of 1.5mm thickness. The gel was allowed to set in a cold room at 4 °C for at least 1 hour and was then placed on a piece of plastic film in a humid chamber until required. After running of the IEF gel, the overlay gel was carefully placed on top of the IEF gel and allowed to stand for 10 minutes. The overlay was removed and transferred to a plastic lunchbox containing staining solution, and incubated in the light with gentle agitation for 30 minutes. Enzyme bands were seen as pale zones on a blue background. The gel was documented by photography and the distance of isoform bands with reference to the cathode was measured.

2.7.16 DNA sequencing and analysis

DNA sequencing reactions were made up in MilliQ water to contain 200-500 ng of plasmid DNA or 30-90 ng of PCR product, with 3-10 pmol of appropriate primer. Primers were designed using the Primer Design programme (version 4, Scientific and Educational Software, 1994) to the following specifications- 17-25 bp, Tm > or = 50 °C, GC content 40 – 60%, no repeats – particularly of G or Cs at the 3' end.

Sequencing was performed in house at the university of Bath on an ABI 337 automated dye primer sequencer. Subsequent DNA searches and analyses were carried out using the following unix, personal PC or Web based programmes:

GELSTART, PILEUP, CLUSTALW and FASTA subroutines of telnet gnome GCG package (Devereux *et al.* 1984), Chromas v. 1.43 (McCarthy 1997), GeneDoc v.1.0.011 (Nicholas and Nicholas 1997), Treecon W (Van de Peer and De Wachter 1994), Clonemanager (Scientific and Educational Software, 1995), National Centre for Biotechnology Information NCBI (www.ncbi.nlm.nih.gov/), ORF finder

(www.ncbi.nlm.nih.gov/gorf/orfig/cgi), PSORT prediction of protein localization sites (www.psort.nibb.ac.jp), PredictProtein (www.embl.heidelberg), SignalP V2 software (http://genome.cbs.dtu.dk/htbin/nph-webface) (Nieilsen *et al.* 1997), ProtParam tool (http://expasy.cbr.nrc.ca/cgi-bin/protparam).

2.8 Reagents and solutions:

Details of chemicals, including catalogue numbers if available, are given under section 2.9 (Chemicals and equipment).

2.8.1 Preparation and transformation of *E.coli*

IPTG stock solution

0.1M isopropylthiogalactoside made up in MilliQ water and sterilised by filtration. Stored at -20°C.

X-Gal stock solution

2% (w/v) 5-chloro-4-bromo-3-indoyl-β-d-galactoside made up in di-methyl formamide. Stored at -20 °C.

Ampicillin

50mg/ml ampicillin (sodium salt) dissolved in MilliQ water. Sterilised by filtration. Stored at -20°C.

2.8.2 Plasmid DNA isolation

Solution I

Glucose	50mM
Tris.HCl (pH 8.0)	25mM
EDTA (pH 8.0)	10mM

Made up in MilliQ water and autoclaved at 10^5 Nm^{-2} for 20 minutes. Stored at 4 °C.

Solution II

1% (w/v) SDS made up in 0.2N NaOH (freshly diluted from a 10N stock).

Solution III	per 100ml
5M Potassium acetate	60ml
Glacial acetic acid	11.5ml
MilliQ water	28.5ml

The resulting solution is 3M with respect to potassium and 5M with respect to acetate.

Phenol: chloroform: isoamyl alcohol

Phenol: chloroform: isoamyl alcohol (25:24:1). Prepared using Tris. equilibrated phenol (pH 7.9) and stored in a light tight container at 4 °C.

Dnase free RNase

Pancreatic RNase (RNaseA) dissolved at a concentration of 10mg/ml in 10mM Tris. HCl (pH 7.5),15mM NaCl. Sterilised by filtration. Heated to 100°C for 15 minutes and allowed to cool slowly to RT. Dispensed into aliquots and stored at -20°C

2.8.3 Electrophoresis of DNA on agarose gels

50X TAE (Tris Acetate EDTA) buffer	per litre
Tris.base	242g
Glacial acetic acid	57.2ml
0.5M EDTA (pH 8.0)	100ml

1X TAE

Prepared by dilution from 50X TAE with distilled water.

Gel loading buffer (Type IV)

Bromophenol blue	0.25% (v/w)
Sucrose	40% (w/v)

Prepared in MilliQ water.

Stored at RT.

Ethidium bromide stock solution

1mg/ml ethidium bromide added to MilliQ water and mixed for several hours on a shaker table. Stored at 4 °C in a light tight container.

2.8.4 Restriction digestion of plasmid and phage DNA

0.5M EDTA (pH 8.0)

EDTA (Ethylenediamine tetra-acetic acid disodium salt) dissolved with stirring in MilliQ water. The pH was adjusted with 2N NaOH and the solution sterilised by autoclaving. The salt did not dissolve completely until the pH reached pH 8.0.

2.8.5 Ligation reactions

Vector DNA

Vector DNA was prepared by restriction digestion of pBluescript KS, followed by either ethanol precipitation (section 2.7.9.2) or gel purification (section 2.7.3.3) in order to purify the DNA from contaminating restriction enzyme protein. For some ligations *Eco*RI cut de-phosphorylated pUC18 (*Eco*RI/BAP Pharmacia Biotech), or pGEM T-easy (Promega) vector DNAs were used.

2.8.6 cDNA library screening

2.8.6.1 Preparation of plating cells and replica plaque lifts

10 mM $MgSO_4$

0.246g of $MgSO_4.7H_20$ dissolved in 100ml of MilliQ water and sterilised by autoclaving at 10^5 Nm^{-2} for 20 minutes.

SM Buffer	per litre:
NaCl	5.8g
$MgSO_4.7H_2O$	2g
Tris.base	6.05g
Gelatin	5ml

Made up with MilliQ water and sterilised by autoclaving at 10^5 Nm^{-2} for 20 minutes.

Denaturing Solution

NaOH	0.5M
NaCl	1.5M

Neutralising Solution

Tris.HCl (pH 7)	0.5M
NaCl	1.5M

2.8.6.2 Hybridisation with heterologous radiolabelled probes (Method 1)

Pre - Washing Solution

5X SSC (v/v)

0.5% SDS (v/v)

Prehybridisation / Hybridisation Solution

5X SSC

5X Denhardts

0.5% SDS (w/v)

Heterologous DNA

Fragmented herring sperm DNA (Sigma) dissolved in sterile MilliQ water at a concentration of 10mg/ml.

100X Denhardts Solution

2% (w/v) BSA Fraction V

2% (w/v) PVP 40 (Polyvinyl-pyrrolidone)

2% (w/v) Ficoll 400,000

Made up with MilliQ water and stored at -20 °C

20X SSC Stock Solution (pH 7.0)

NaCl	3M
Tri-sodium citrate	0.3M

Dissolved in 400ml MilliQ water, pH adjusted to 7.0 with NaOH and the volume made up to 500ml. May be autoclaved for long-term storage.

2.8.6.3. Hybridisation with radiolabelled probes (Method 2)

Pre-hybridisation/hybridisation solution

Sodium phosphate (pH 7.2)	250mM
EDTA (pH 8)	1mM
SDS	7% (w/v)

Made up by dissolving sodium phosphate in 900ml distilled water. The pH was adjusted with orthophosphoric acid and the other components added and dissolved by stirring with a magnetic flea prior to making up the volume to 1l.

Low stringency wash solution

Sodium phosphate (pH 7.2)	125mM
SDS	1% (w/v)

Medium stringency wash solution

Sodium phosphate (pH 7.2)	63mM
SDS	0.5% (w/v)

High stringency wash solution

Sodium phosphate (pH 7.2)	15mM
SDS	0.5% (w/v)

2.8.6.3 Removal of unincorporated nucleotides

Column buffer

NaCl	100mM
Tris.HCl (pH 7.5)	10mM
EDTA (pH 8.0)	1mM

Made up in MilliQ water and sterilised by autoclaving at 10^5 Nm^{-2} for 20 minutes.

Sephadex G-50

Sephadex G-50 (fine) added to column buffer to form a slurry. Sterilised by autoclaving at 10^5 Nm^{-2} for 20 minutes.

2.8.7 Polymerase Chain Reaction

PCR Amplification Master Mix - (sufficient for 12 reactions)

		Final concentration
68 µl	Sterile MilliQ	---
12 µl	Taq Buffer (10x)	1x
12 µl	2mM dNTP mix*[1]	200µM
12 µ l	1 µ M forward lambda primer *[2]	0.1µM
12 µ l	1 µ M reverse lambda primer *[2]	0.1µM
2 µ l	50mM $MgCl_2$	0.83mM
2 µ l	Taq Polymerase	10 units
120µl		

*[1]diluted from 50mM stocks (Bioline) e.g. to prepare 100µl of 2mM dNTP mix, 4 µl of each dNTP was added to 84 µl sterile MilliQ and stored at -20 °C

*[2] Prepared by dilution from stock primer

2.8.8 Large scale phage DNA extraction

RNase

Pancreatic RNase (RNaseA) dissolved at a concentration of 10mg/ml in 10mM Tris. HCl (pH 7.5), 15mM NaCl. Sterilised by filtration. Heated to 100°C for 15 minutes and allowed to cool slowly to RT. Dispensed into aliquots and stored at -20°C

DNase I

10mg/ml in 0.1.5M NaCl and 50% glycerol. Sterilised by filtration and stored in 1ml aliquots at -20°C

Proteinase K

20mg/ml made up in MilliQ and sterilised by filtration. Stored at -20°C.

0.5M EDTA (pH 8.5)

Made up in MilliQ water and sterilised by autoclaving at 10^5 Nm^{-2} for 20 minutes.

10% SDS

Made up with MilliQ water.

Phenol: chloroform: isoamyl alcohol

Phenol: chloroform: isoamyl alcohol (25:24:1) prepared using equilibrated phenol (pH 7.9) and stored at 4°C in a light tight container.

3M Sodium Acetate (pH 6.0)

Made up in MilliQ water and sterilised by autoclaving at 10^5 Nm^{-2} for 20 minutes.

95% Ethanol - prepared by dilution from absolute alcohol using MilliQ water.

70% Ethanol - prepared by dilution from absolute alcohol using MilliQ water.

2.8.9 Genomic DNA Isolation and Southern blotting

Dellaporta extraction buffer

Tris. HCl	100mM
EDTA	50mM
NaCl	500mM

Autoclaved and stored at room temperature. Just before use β-mercaptoethanol and polyvinylpyrolidine were added at a final concentration of 0.07% and 1% respectively.

Ethanol precipitation

Salt	Stock solution	Final concentration	Volume used
sodium acetate	3.0 M (pH 5.2)	0.3 M	0.1
ammonium acetate	10 M	2.5M	0.25

Spermidine

1 M stock prepared in MilliQ water, sterilised by filtration and stored at -20 °C

Depurinating solution

0.25M HCl prepared by dilution in distilled water.

Denaturing solution

NaOH	0.5M
NaCl	1.5M

Neutralising solution

Tris.HCl (pH 7)	1.5M
NaCl	1.5M

Southern blot hybridisation/pre-hybridisation solution

Sodium phosphate (pH 7.2)	250mM
EDTA (pH 8)	1mM
SDS	7% (w/v)
PEG 6000	4%(w/v)

Made up by dissolving sodium phosphate in 900ml distilled water. The pH was adjusted with orthophosphoric acid and the other components added and dissolved by stirring with a magnetic flea prior to making up the volume to 1l.

2.8.10 RNA Isolation and Northern Blotting

2.8.10.1 Cassava root RNA extraction

Extraction buffer

Tris.HCl (pH7.5)	100mM
NaCl	100mM
EDTA	25mM
Sodium Laurylsarcosine	1% (w/v)
PVP K30	2% (w/v)
*β-mercaptoethanol	2% (v/v)

Glassware was treated at 180 °C for at least 4 hours. MilliQ water and stock solutions (5M NaCl, 0.5M EDTA) were treated with either 0.1% DEPC or 10% DMPC stock solution for at least 1 hour and were autoclaved. . For Tris a new or reserved batch for RNA work was used and a 0.5M stock prepared in RNase free water. Other chemicals were added as powder

*Added just before use.

Chloroform

Chloroform:isoamyl alcohol (24:1). A reserved stock for RNA work was used.

Phenol: chloroform: isoamyl alcohol

Phenol: chloroform: isoamyl alcohol (25: 24: 1). Stored at 4 °C in a light tight container. A reserved stock for RNA work was used. In order to minimise co-isolation of DNA, acid phenol (pH 6.6) was used for all RNA work since at pH <7 DNA partitions into the organic phase whilst RNA remains in the aqueous phase.

8M LiCl

Made up with MilliQ water, treated with DEPC or DMPC and autoclaved at 10^5 Nm^{-2} for 20 minutes.

DEPC treated water/solutions

Working in a fume hood, DEPC (diethyl pyrocarbonate) was added to a final concentration of 1% (v/v). The solution was mixed and allowed stand for 1 hour before autoclaving at 10^5 Nm^{-2} for 20 minutes

DMPC treated water/solutions

Stock DMPC solution was prepared by dissolving 1% (v/v) DMPC (dimethyl pyrocarbonate) in a 50% ethanol: MilliQ solution. Working in a fume hood, 10% (v/v) of the DMPC stock was added to the solution for treatment. The solution was mixed and allowed stand for 1 hour before autoclaving at 10^5 Nm^{-2} for 20 minutes.

2.8.10.2 Cassava leaf RNA extraction

Extraction buffer

Tris.HCl (pH7.5)	100mM
NaCl	100mM
EDTA	20mM
Sodium Laurylsarcosine	1% (w/v)

Glassware was treated at 180 °C for at least 4 hours. MilliQ water and stock solutions (5M NaCl, 0.5M EDTA) were treated with either 0.1% DEPC or 10% DMPC stock solution for at least 1 hour and were autoclaved. For Tris a new or reserved batch for RNA work was used and a 0.5M stock prepared in RNase free water. Other chemicals

were added as powder

3M Sodium acetate (pH 4.8)

Made up with MilliQ water, treated with DEPC or DMPC and autoclaved at 10^5 Nm^{-2} for 20 minutes.

2.8.10.3 Northern blotting and hybridisation

5X MOPS buffer

MOPS (pH 7.0)	0.2M
Sodium acetate	0.05M
EDTA (pH 8.0)	0.005M

To prepare 2 litres of buffer 83.72g of MOPS free acid (N-morpholino-propanesulphonic acid) and 8.23g of sodium acetate were dissolved in 1.6l of MilliQ water and stirred until dissolved. 20ml of 0.5M EDTA was added and the pH adjusted to 7.0 with 10M NaOH. The volume was adjusted and the solution treated with DEPC or DMPC before autoclaving at 10^5 Nm^{-2} for 20 minutes. After autoclaving the solution turned a pale yellow colour, but this did not affect the quality of the buffer.

1X MOPS running buffer

Prepared by dilution of 5X MOPS buffer with DEPC or DMPC treated water.

RNA loading buffer

Glycerol	50%
EDTA (pH8)	1mM
Bromophenol blue	0.4%

Prepared in DEPC or DMPC treated water and dispensed in 0.5ml aliquots. Stored at –20°C. For loading buffer containing ethidium bromide, 95µl of loading buffer was mixed with 5µl of 1mg/ml ethidium bromide prepared in RNase free water. The final concentration of ethidium bromide was 0.05µg/µl.

Denaturing formaldehyde gel

Gels were prepared by dissolving agarose (Type III technical) at a concentration of 1.5% (w/v) in RNase free water containing 5X MOPS buffer to a final concentration of 1X. After cooling to 60°C, formaldehyde (37% solution) was added to a final

concentration of 2.2M. After pouring into an RNase free gel tray with appropriate comb, gels were allowed to set for at least 30 minutes in a fume cupboard.

Northern hybridisation/Prehybridisation solution

Phosphate buffer (pH7.2)	0.5M
Skimmed milk (fat free)	1% (w/v)
SDS	7% (w/v)

To prepare 500ml hybridisation solution, 171ml of a 1M stock of Na_2HPO_4 prepared in DEPC treated water was combined with 79ml of a 1M stock of NaH_2PO_4 prepared in DEPC treated water. The pH was checked and if necessary adjusted with 10M NaOH to exactly 7.2. The volume was adjusted to 500ml, the dry componants added and the solution stirred with a magnetic flea.

Low stringency wash solution

1X SSC (v/v)

0.1% SDS (w/v)

Prepared in DEPC or DMPC treated water.

High stringency wash solution

0.2X SSC (v/v)

0.2% SDS (w/v)

Prepared in DEPC or DMPC treated water.

2.8.11 *In vivo* detection and localisation of reactive oxygen species

2.8.11.1 Detection of superoxide by vacuum infiltration with NBT

K_2HPO_4 buffer

10mM K_2HPO_4 (pH 6) made up in MilliQ water.

Chloral hydrate

Chloral hydrate (4g/ml) in MilliQ water.

2.8.11.2 *In situ* detection of hydrogen peroxide by vacuum infiltration with DAB

DAB reagent

DAB (3,3 diaminobenzidine tetrahydrochloride) dissolved at a concentration of 2mg/ml (w/v) in MilliQ water.

DAB and ascorbate control reagent

DAB (3,3 diaminobenzidine tetrahydrochloride) dissolved at a concentration of 2mg/ml (w/v) in 10mM ascorbate.

2.8.12. In vitro quantification of hydrogen peroxide

5% Metaphosphoric acid

Metaphosphoric acid (35%) dissolved in MilliQ water to a final concentration of 5% and stored at 4°C.

Tris buffer

0.2M Tris.HCl (pH 8.5) prepared in MilliQ water.

Potassium ferricyanide

5mM potassium ferricyanide made up in Tris buffer.

Luminol

0.01mM luminol prepared by serial dilution in DMSO from a 0.1M stock prepared in DMSO. Stocks and dilutions were freshly prepared each day and protected from light before use.

Hydrogen peroxide stock solution

Prepared by dilution of 0.1M (30%) solution to a final concentration of 1mM in 5% metaphosphoric acid. Stock solution was freshly prepared each day.

Dowex basic anion exchange resin

A slurry was prepared by covering resin in a 50ml tube with an equal volume of deionised water. The powder was dispersed by gentle inversion for 1 minute and allowed to settle for 15 minutes. The upper water layer was discarded and replaced with

a fresh volume of deionised water. The slurry was stored at RT until required.

2.8.12 TLC separation and detection of antioxidant compounds

DPPH staining reagent

DPPH (1,1 Diphenyl-2-picryl-hydrazl) 1mg/ml (w/v) in methanol. Freshly prepared.

2.8.14 Tissue print and in vivo detection and localisation of enzyme activity

2.8.14.1 Tissue print detection and localisation of catalase activity

Solution A

60mM Sodium thiosulphate.

Dissolved in MilliQ water and stored at RT

Solution B

3% (v/v) H_2O_2 in MilliQ water. Freshly prepared.

Solution C

Potassium Iodide	90mM
Glacial acetic acid	0.5% (v/v)

2.8.14.2 Tissue print detection and localisation of peroxidase activity

Detection solution

H_2O_2	0.1%
aqueous guiacol	10mM

Made up in 50mM phosphate buffer pH 5.2

2.8.14.3 Tissue print detection and localisation of total protein

Coomassie blue stain

Coomassie blue (Brilliant blue R Sigma)	0.1% (w/v)
Methanol	20 % v/v

Acetic acid	7% v/v

Made up with distilled water and stored at room temperature.

Coomassie blue de-stain

Methanol	50 % v/v
Acetic acid	7% v/v

Made up with distilled water and stored at room temperature.

2.8.14.4 Phloroglucinol staining of cassava root tissue

Phloroglucinol stain reagent

5% Phloroglucinol in methanol. Stored at room temperature.

Phloroglucinol de-stain

HCl mixed with MilliQ water in a 1:1 ratio.

2.8.14.5 Toluidine blue staining of cassava root tissue

Toluidine blue reagent

Toluidine blue 1% (w/v) prepared in 0.05% citrate phosphate buffer (pH 4).

Citrate phosphate buffer (pH 4)

0.05% Na_2HPO_4 dissolved in MilliQ water. The pH was adjusted using citric acid.

2.8.15 Protein extraction and analysis

2.8.15.1 Protein extraction and electrophoresis

Cassava root protein extraction buffer

Potassium phosphate buffer (pH 6.4)	0.1M
DTT (Dithiothreitol)	1mM
EDTA	1mM

Dialysis tubing preparation solution

$NaHCO_4$	100mM
EDTA	10mM

Prepared with MilliQ water

Coomassie blue G Reagent

	Per l
Coomassie brilliant blue G	100mg
Orthophosphoric acid (85%)	100ml
Ethanol (95%)	50ml

Dissolved by stirring at RT for several hours, and slowly made up to 1l with MilliQ water. Placed at 4 °C overnight to precipitate, and centrifuged at 8000rpm in a Sorvall GSA rotor at 4 °C. Transferred to a fresh container and stored in the dark at 4 °C for up to 6 months.

Cathode solution

0.1M NaOH freshly prepared with MilliQ water and stored in a tightly sealed bottle for no longer than 1 week.

Anode solution

0.4M aspartic acid and stored in a tightly sealed bottle for no longer than 1 week.

2.8.15.2 Staining of pI marker

Fixing solution

TCA (Trichloroacetic acid)	29g
Sulphosalicylic acid	8.5g

Made up to 250ml with MilliQ water and stored for up to 3 months at RT

Staining solution

0.5% Coomassie Blue R dissolved in de-staining solution. Heated to 60 °C for 1 hour and filtered through Whatman filter paper.

De-staining solution

Absolute ethanol	250ml
Acetic acid	80ml

Made up to 1l and stored for no longer than 3 weeks at RT

Preserving solution

25ml glycerol made up to 250ml with de-staining solution.

2.8.15.3 Detection of Peroxidase isoforms on IEF gels

Detection solution

H_2O_2	0.1%
aqueous guiacol	10mM

Made up in 50mM phosphate buffer pH 5.2

2.8.15.4 Detection of Superoxide Dismutase on protein gels

Overlay gel

1.5% agarose in 50mM Tris.HCl buffer (pH 8.5)

Staining solution

50mM Tris.HCl (pH 8.5)	80ml
NBT (Nitroblue tetrazolium)	10mg
PMS (Phenazine methosulphate)	6mg
$MgCl_2.6H_2O$	15mg

2.9. Chemicals and Equipment

2.9.1 Chemicals

α ^{32}P dCTP isoblue. 39011X2. ICN.

Acetic acid glacial. AnalaR grade. BDH. 10001.

Agar. Type III technical. Oxoid.

Agarose. Ultra-pure electrophoresis grade. Gibco BRL. 15510-027.

Ampicillin (D [-]-a-Aminobenzyl penicillin) Sodium salt. Sigma. A9518.

Ampholine PAG plates pH 3.5 – 9.5. Pharmacia 80-1124-80

Ascorbate. L-ascorbic acid. Sigma. A7506.

Autoradiographic film. Kodak X-Omat AR 18 X 24cm . 8532665.

Autoradiographic film. Kodak X-Omat LS 35 X 43cm. 8926024

Brilliant blue R. Sigma. BO149.

Bromophenol blue. BDH. 6239180.

BSA (Bovine serum albumin). Fraction V. Sigma. A 6793).

Casein Hydrolysate. BDH. 44021.

Catalase. From *Aspergillus niger*. Sigma. C-3515

Catechin. A kind gift from Dr. H. Buschmann.

Chloral hydrate. BDH. 0524640.

Chloroform. ACS Reagent. Sigma. C5312.

Coomassie blue (Brilliant blue R).Sigma. B0149.

Cupric nitrate. Sigma. C2646.

DAB (3,3 diaminobenzidine tetrahydrochloride). Sigma. D637.

Dialysis tubing, size 2, MWCO 12-14000 Da. Medicell International.

DNase I. Sigma. DN25

DNA ladder. 100bp ladder. Pharmacia. 27-4001-01

DEPC. Diethyl pyrocarbonate. Sigma. D5758.

DMPC (Dimethyl pyrocarbonate). Sigma. D5520.

DMSO (Dimethyl sulphoxide). Sigma. D-8779.

DPPH (1,1 Diphenyl-2-picryl-hydrazl). Free radical. Sigma. D-9132

DTT (Dithiothreitol) Sigma. D-8161.

Dowex strongly basic anion exchanger. Sigma. 1X8 400.

EDTA (Ethylenediamine tetra-acetic acid disodium salt).AnalaR grade.BDH. 100935V.

Esculetin. A kind gift from Dr. H. Buschmann.

Ethidium bromide. Sigma. E8751.

Ethyl acetate. HPLC grade. Rathburn Chemicals Ltd.

Ficoll 400,000. Sigma. F2637.

Formaldehyde. 37% solution. Fluka. 47630.

Formamide. Sigma. F5786.

Formic acid. 99%. Sigma. F-0507.

Fragmented herring sperm DNA. Deoxyribonucleic acid, degraded free acid from Herring sperm. Sigma. D3159.

Gallocatechin. A kind gift from Dr. H. Buschmann.

Gelatin. Gelatin for Microbiology. Merck.

Glucose. D (+) glucose. AnalaR grade. BDH. 101176K

Glycerol. AnalaR grade. BDH. 101184K.

Guiacol. (2-methoxyphenol). Sigma. G5502.

Hydrochloric acid. AnalaR grade. BDH. 10125.

Hydrogen peroxide. 30% solution. Sigma. H1009.

IPTG (isopropylthiogalactoside) Dioxan Free. Melford labs Ltd. MB1008.

IEF Gels. Ampholine PAG plate pH3.5 – 9.5. Amersham Pharmacia. 80-1124-80

Jasmonic acid. Sigma. J2500

Isoamyl alcohol. ACS Reagent. Sigma. I0640

Lambda forward primer .NEB. 1231

Lambda reverse primer NEB. 1232.

Lithium chloride anhydrous. AnalaR grade. BDH. 10374.

Luminol. (5-Amino-2,3-dihydro-1,4-phthalazinedione). Sigma. A8511.

Magnesium sulphate. $MgSO_4.7H_20$. BDH. 29117.

Maltose. Maltose monohydrate. Sigma. M5895.

β-mercaptoethanol. (2-mercaptoethanol). Sigma. M6250.

Methanol. Absolute, acetone free. Sigma. M1775.

Metaphosphoric acid (35%). ACS reagent. Sigma. M5043.

Methylene blue. Fischer. M/P206/46.

Miracloth. Calbiochem. 475855.

MOPS. (3-(N-Morpholino)propanesulphonic acid. Biochemical grade. BDH. 443832T.

NBT. Nitroblue tetrazolium. Sigma. N6876.

Orthophosphoric acid 88%. AR grade. Fissons.

pI Marker. Pharmacia Biotech. 11-B-045-11

PEG (Polyethylene glycol) 6000. BDH. 44271.

PEG (Polyethylene glycol) 8000. Sigma. P-2139.

PEG (Polyethylene glycol) 35,000. Fluka Chemika. 81310.

Phenol. Saturated phenol pH 6.6/ 7.9. BDH. 436754G

Phloroglucinol (1,3,5 Trihydroxybenzene). Sigma. P3502.

Photographic film. Kodak Ektachrome 35mm colour slide film 160T.

PMS. Phenazine methosulphate. Sigma. P-9625.

Potassium ferricyanide. SLR grade. Fissons.

Potassium Iodide. AnalaR grade. BDH. 10212.

di-Potassium hydrogen orthophosphate anhydrous (K_2HPO_4). Fischer. P/5245/53.

Potassium acetate. AnalaR grade. BDH. 103504X.

Proteinase K. Sigma. P2308.

PVP. (Polyvinyl pyrrolidone). Sigma. PVP-40

PVP. (Polyvinyl pyrrolidone insoluble) Polyclar AT. BDH.

RNA ladder. NEB. 362.

RNase. Ribonuclease A. Sigma. R5000.

"RNase Zap". Ambion. 9780.

Scopoletin. A kind gift from Dr. H. Buschmann.

Scopolin. A kind gift from Dr. H. Buschmann.

SDS. (Sodium dodecyl sulphate). Biochemical grade. BDH. 444464T.

Sephadex G-50 (fine). Pharmacia. 5521.

Skimmed milk (fat free). BBL Becton dickenson. 11915.

Sodium acetate anhydrous. AnalaR grade. BDH. 102365R.

Sodium chloride (NaCl).AnalaR grade. BDH. 10241K.

Sodium hydroxide. AnalaR grade. BDH. 102525P.

Sodium laurylsarcosine. (N-Laurylsarcosine) Sodium salt. Sigma. L9150.

Sodium phosphate. Dibasic anhydrous. Sigma. S7907.

Sodium dihydrogen orthophosphate. AnalaR grade. BDH. 30132.

di-Sodium hydrogen orthophosphate. AnalaR grade. BDH. 102494C.

Sodium thiosulphate. AnalaR grade. BDH. 10268.

Spermidine. (N-3 aminopropyl-1,4-butanediamine). Sigma. S2501.

Starch. Soluble starch. AnalaR grade. BDH. 0518260.

Sucrose. AnalaR grade. BDH. 10274.

Superoxide dismutase. (from horseradish) . Sigma. S4636.

Taq Polymerase (Bioline)

Tri-sodium citrate. AnalaR grade. BDH. 102425M.

Tris(hydroxymethyl)methylamine. AnalaR grade.BDH. 103156X

TLC plates. HPTLC aluminium sheets 20 X 20cm. Silica Gel 60 F_{254}. Merck.

Thiamine HCl. Sigma. T4625.

Tryptone (pancreatic digest of casein). Difco.

Vector DNA. pUC18 *Eco*RI/BAP. Pharmacia. 27-4854-01.

X-Gal (5-chloro-4-bromo-3-indoyl-β-d-galactoside). Sigma. B4252.

Yeast extract. Difco.

2.9.2 Equipment

Balances:	Sartorius analytic (Fisons Instruments) Sartorius 1213 MPC (Fisons Instruments)
Speed Vac:	Savant Speed Vac Concentrator. Stratatech Scientific
Microscope:	Olympus BH2
Centrifuges:	Sorvall RC5C. Sorvall Instuments. Du Pont. Eppendorf 5415C bench centrifuge
Autoradiograph development:	X-Omat 2X Processor.
DNA sequencing:	ABI 337 Automated Fluorescent Sequencer.
Incubators:	Gallenkamp Cooled Orbital Incubator
Luminometer:	EG and G Berthold Microplate luminometer LB 96V Microlumat Plus with EG and G Berthold Winglow software.
PCR:	PTC 100 Programmable Thermal Controller. MJ Research Inc. GRI.
Photography:	Digital Graphic Printer UPD86OE. Sony with "Grab it" software. 35mm camera with macrolens.
Hybridisation:	Hybaid Hybridisation Oven. Hybaid. Innova 4000 Incubator Shaker. New Brunswick Scientific.
Transilluminator:	UVP White/UV transilluminator. UVP Life Sciences.
Scanner:	Nikon Coolscan III with Nikon Scan 2.1 software HP Scanjet 6200C
Microplate reader:	Dynatech MR5000 microplate reader.
IEF Gels:	LKB BROMMA 2117 Multiphor horizontal electrohporesis apparatus with LKB BROMMA 2197 Power supply and Churchill chiller thermo circulator.

CHAPTER THREE:

- # STUDIES ON THE ROLE OF CATALASE DURING POST-HARVEST PHYSIOLOGICAL DETERIORATION

3.1 Introduction and literature review

3.1.1 Types and classification of catalases

The enzyme catalase serves to catalyse the break down of hydrogen peroxide to water and molecular oxygen and is found in virtually all aerobic organisms. Catalase activity in living tissues was first postulated by Thenard in 1811, who noted that plant and animal tissues could rapidly break down hydrogen peroxide. In 1901 Loew named this hydrogen peroxide degrading enzyme "catalase" and in 1923 Warburg demonstrated that the active centre contained iron, since its activity could be inhibited by cyanide. To date 3 types of catalase have been described – the catalase-peroxidases, the manganese (Mn) catalases and the more common tetrameric heme containing "typical catalases". The catalase-peroxidases show sequence similarity to heme peroxidases rather than to catalases, but exhibit catalatic activity. The Mn catalases utilize manganese, rather than ferric heme at their active site. To date only 3 such Mn catalases have been described and in certain prokaryotes only (Ossowski *et al.* 1993, Bravo *et al.* 1997, Zamocky and Koller 1999). Neither the catalase-peroxidases nor the Mn catalases will be discussed further in this chapter.

The typical heme-containing catalases (H_2O_2: H_2O_2 oxidoreductase EC 1.11.1.6) are tetramers, and in plants may be composed of 4 like (homotetrameric) or unlike (heterotetrameric) monomeric subunits. Animal genomes in general contain a single catalase structural gene (an exception being *Caenorhabditis elegans* which has two). By contrast, in plants, monomeric structural genes are encoded by a small gene family. In well characterised plant systems such as *Arabidopsis thaliana*, maize (*Zea mays*), and tobacco (*Nicotinia plumbaginifolia*) three catalase gene family members have been described (Skadesen and Scandalios 1986, Frugoli *et al.* 1998). The products of the structural genes may associate to form homo- or hetero- tetramers, giving rise to multiple isoforms that may play different physiological roles (Scandalios *et al.* 1997, Frugoli *et al.* 1996). The *Arabidopsis* catalase gene family, for example, contains 3 catalase genes, the products of which associate to form at least 6 isozymes that are resolved by non –denaturing protein electrophoresis. Both the individual mRNAs and the individual isozymes show distinct patterns of organ specific expression (McClung 1997, Frugoli *et al.* 1996).

3.1.2 Properties and reactions catalysed by plant catalase

As proposed by Chance (1973) the behaviour of the enzyme depends on the steady state concentration of hydrogen peroxide. At high hydrogen peroxide concentrations direct dismutation of hydrogen peroxide to water and oxygen occurs (catalatic mode) as below-

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

At low concentrations (< 10^{-6} M) a variety of substrates such as ethanol and ascorbic acid may be oxidised as below (peroxidatic mode)-

$$RH_2 + H_2O_2 \longrightarrow R + 2H_2O$$

In the catalatic mode, catalase has an extremely high Michaelis constant and is not easily saturated with substrate. In both catalatic and peroxidatic modes, a two-step mechanism is used. In the first step, the heme iron of catalase interacts with hydrogen peroxide to form an intermediate oxygen- rich iron peroxide (compound I) –

$$\text{CAT-Fe-OH} + H_2O_2 \longrightarrow \underset{\textbf{compound I}}{\text{CAT-Fe-OOH}} + H_2O$$

At high hydrogen peroxide concentrations compound I reacts with a second molecule of hydrogen peroxide to form water and molecular oxygen –

$$\underset{\textbf{compound I}}{\text{CAT-Fe-OOH}} + H_2O_2 \longrightarrow \text{CAT-Fe-OH} + H_2O + O_2$$

At low hydrogen peroxide concentrations compound I is reduced peroxidatically by a variety of hydrogen donors-

$$\underset{\textbf{compound I}}{\text{CAT-Fe-OOH}} + RH_2 \longrightarrow \text{CAT-Fe-OH} + H_2O + R$$

3.1.3 Location and targeting of plant catalases

In higher plants, most catalase isozymes co-isolate with microbodies, with the exception of maize Cat3 which co-isolates exclusively with mitochondria (Higo and Higo 1996,

Scandalios *et al.* 1997). The major proposed sources and location of hydrogen peroxide production in plants are the chloroplasts (via leakage from the electron transport chain of photosystem I); the mitochondria (via leakage from the respiratory electron transport chain); and within microbodies (via catabolic oxidation reactions). In addition, hydrogen peroxide generated in these subcellular components may diffuse to other locations. To date no catalase activity has been associated with chloroplasts and scavenging of hydrogen peroxide is believed to be effected by ascorbate peroxidase via the Halliwell-Asada cycle. However, scavenging of hydrogen peroxide within the cytosol, mitochondria and particularly in microbodies, is believed to be effected by catalase (Scandalios 1997). Microbodies are membrane bound vesicles (0.3 – 1.5μm diameter), which arise from the golgi body of both plant and animal cells. They are characterised by the presence of flavin linked oxidases which produce hydrogen peroxide, and catalase which subsequently degrades the hydrogen peroxide (Hall *et al.* 1982). Two to four types of plant microbody, containing a differing complement of enzymes and specialised for differing functions, have been described – peroxisomes, glyoxisomes, unspecialised microbodies and microbodies for the production of ureides in uninfected cells of nodules in legume roots. Peroxisomes are located in green/ photosynthetic tissues and are involved in photorespiration i.e. the oxidation of carbohydrates in the presence of light and high levels of oxygen; and the breakdown of purines. Characteristic enzymes found in peroxisomes include glycolate oxidase and hydroxypyruvate reductase. Glyoxisomes are specialised peroxisomes found in storage tissues and senescing organs that are involved in mobilisation of storage lipids to carbohydrates. Characteristic enzymes are those of the glyoxylate cycle and fatty acid β-oxidation such as isocitrate lyase, citrate synthase, malate synthase and malate dehydrogenase. Unspecialised microbodies whose biological function has not been characterised have also been described in the roots of higher plants, whilst microbodies containing uricase and specialised for ureide synthesis have been detected in legume root nodules (Esaka *et al.* 1997, Hall *et al.* 1982, Lea and Leagood 1999, Olsen, 1998).

Catalase apo-protein monomeric subunits are nuclear encoded and are translated on free ribosomes in the cytoplasm. Export from the cytoplasm is believed to occur via a receptor mediated transport process, followed by intra-organellar association of each apo-monomer with its heme moiety and assembly of functional protein tetramers as below-

Step 1: apo-monomer (M) + heme (H)	→	holo-monomer (MH)
Step 2: (MH) + (MH)	→	holo-dimer $(MH)_2$
Step 3: $(MH)_2$ + $(MH)_2$	→	holo-tetramer $(MH)_4$

(Bravo *et al.* 1997). Post-translational transport to microbodies is proposed to occur via interaction with a PTS (Peroxisomal Targeting Signal) receptor (Mullen *et al.* 1997, Zamocky and Koller 1999). At least 2 different types of peroxisomal targeting signal (PTS) -designated PTS1 and PTS2 - have been described in peroxisome-targeted proteins. Of these, the carboxy terminal tripeptide PTS1 motif (consensus Cys/Ala/Ser/Pro - Lys/Arg- Ile/Leu/Met) located at the carboxy terminus is most common (Mullen *et al.* 1997, Hayashi *et al.* 1997) and is found in most but not all plant, animal and yeast peroxisomal proteins. The less frequently used nona-peptide PTS2 is located at or near the amino terminus of the peroxisomal protein, and has the consensus sequence Arg/Lys - Leu/Ile/Gln/Val - X_5 - His/Gln – Leu/Ala.

There is however some dispute as to the microbody-targeting signal for catalase. Catalases are unusual in that the carboxy-terminal tripeptide does not constitute a typical PTS1, nor is an amino-terminal PTS2 present. An internal Ser-Lys-Leu type motif (consensus Ser- Lys/Arg- His/Leu) (Esaka *et al.* 1997) located 9 residues from the carboxy terminus is often proposed as the microbody targeting signal for plant catalases on the basis of sequence conservation (Frugoli *et al.* 1996, Skadesen *et al.* 1995, Suzuki *et al.* 1994, Abler and Scandalios 1993). This motif is highly similar to the PTS1 found in other yeast and mammalian peroxisomal proteins (consensus Ser/Ala/Cys- Lys/Arg/His - Leu/Met/Phe) (Mullen *et al.* 1997); and the plant PTS1 (consensus Cys/Ala/Ser/Pro- Lys/Arg- Ile/Leu/Met) found in other plant peroxisomal proteins such as isocitrate lyase, malate synthase and uricase (Hayashi *et al.* 1997). However, in these cases the tripeptide is located at the carboxy terminus rather than internally, and the carboxy terminal location is critical for function (Olsen 1998). In addition, Trelease *et al.* (1996) found that the internal Ser-Lys-Leu motif of rat liver catalase was not functional, whilst a carboxy terminal tripeptide (Ala-Asn-Leu) was necessary and sufficient for peroxisomal targeting. Mullen and colleagues propose an alternate tripeptide (consensus Pro - Ser/Thr/Asn - Met/Ile), which is also conserved among plant catalases and is located at the carboxy terminus, as the peroxisomal targeting signal for plant catalases. In immunofluorescence localisation studies of transiently-expressed epitope-tagged constructs in tobacco cell cultures the Pro-Ser-Ile motif (consensus Pro - Ser/Thr/Asn - Met/Ile), was necessary but not sufficient for peroxisomal targeting and

was context dependant, requiring the presence of upstream accessory residues such as Ser-Arg-Leu-Asn-Val-Arg amino terminal to the Pro-Ser-Ile motif.

3.1.4 Previous studies in cassava and the role of catalase in other plant systems

A possible role for catalases during post-harvest physiological deterioration of cassava storage roots was first postulated by Czyhrinciw and Jaffe (1951), who proposed catalases, peroxidases and dehydrogenases as candidate enzymes. Work by Averre (1967) confirmed that the response was enzymatic in nature, however aside from the increase in catalase activity noted by Czyhrinciw and Jaffe (1951), no further studies on catalase during cassava PPD have been carried out.

The catalases of higher plants play diverse roles in resistance to oxidative stress, germination (via scavenging of H_2O_2 produced during the β-oxidation of lipids to sugars), and photorespiration (via scavenging of H_2O_2 produced during the photorespiratory pathway) (Frugoli *et al.* 1998). In addition, they have been proposed to play a role in mediation of signal transduction involving H_2O_2 as a second messenger (Ryals ***et al.*** 1995, Low and Merida 1996, Anderson *et al.* 1998).

Regulation of plant catalases occurs in a complex tissue and developmentally specific manner, and in addition may be modulated by environmental signals including light and temperature stress (Esaka *et al.* 1983, Guan and Scandalios 1995, Suzuki *et al.* 1997). Differential regulation in response to wounding and senescence has also been reported. In pumpkin cotyledons 3 catalase mRNAs showed differing expression patterns during senescence with increased expression of *Cat1* but decreased expression of *Cat2* and *Cat3* (Esaka *et al.* 1997). In *Brassica napus* several cDNAs with enhanced expression during leaf senescence were isolated by a subtractive hybridisation approach and included a catalase transcript - LSC 650)(Buchanan-Wollaston and Ainsworth 1997); whilst analysis of tomato leaf senescence related cDNAs revealed a catalase - SEND 36 - which was down-regulated during senescence (John *et al.* 1997). Wounding of sweet potato tuberous roots led to increased catalase transcript expression after a lag of ~10 hours and with a peak activity occurring ~30 hours after wounding (Sakajo *et al.* 1987). The authors propose a sequential mechanism for the increase in catalase protein activity. Following the lag period (~10 hours after wounding) catalase mRNA levels increased and thus enhanced enzyme activity was attributed to increased transcription (and / or decreased transcript degradation). However during the early stages after wounding the amount of catalase mRNA associated with ribosomes increased despite no significant increase in total catalase mRNA levels. The authors propose that in the initial stages

after wounding catalase activity increased via increased translation. In wounded pumpkin cotyledons, Cat1 and Cat2 transcript levels were induced, whilst Cat1 transcript levels were unaffected (Esaka *et al.* 1997).

Recently, catalase has been implicated in defence signal transduction in higher plants, particularly in the context of the HR (hypersensitive response) and SAR (systemic acquired resistance). Chen *et al.* (1993) reported a salicylic acid binding protein (SABP) that was characterised as a salicylic acid inhibited catalase. Subsequently SABP and salicylic acid inhibited catalase activities were reported for several plants including *Arabidopsis*, tomato and cucumber (Sanchez-Casas and Klessig 1994). It was proposed that these SABP catalases were a receptor for salicylic acid and were involved in defence signal transduction. The binding of salicylic acid to catalase, resulting in enzyme inhibition, should allow an increase in hydrogen peroxide levels which result in activation of PR genes and other defence related genes, possibly via a ROS or redox activated transcription factor analogous to mammalian NF-κB. This proposal has been challenged by Ruffner *et al.* (1995) who found that salicylic acid bound specifically to iron containing enzymes including catalase, aconitase, lipoxygenase and peroxidase from both plant and animal sources. In addition they suggest the concentrations required for catalase inhibition (10^{-3} M) would rarely if ever be attained *in planta,* although they do disregard compartmentation. Further studies in other plant systems failed to detect increased levels of H_2O_2 in tissues undergoing SAR, or found that SA did not act as a catalase inhibitor in some plant systems (Ruffner *et al.* 1995, Ryals *et al.* 1995, Tenhaken and Rubel 1997, Dat *et al.* 1998).

An alternate model proposed by Anderson and colleagues (1998), suggests that interaction of catalase with salicylic acid results not only in accumulation of H_2O_2, but also SA radicals, which may be produced when SA acts as an electron donor for the peroxidative cycle of catalase. Such SA radicals were found to induce lipid peroxidation in tobacco cell cultures, and the authors suggest that one or more lipid peroxidation products may function downstream to mediate signal transduction.

A third model proposes that generation of molecular oxygen via catalase mediated breakdown of H_2O_2 fuels production of SA from benzoic acid (Leon *et al.* 1995). Consistent with this interpretation H_2O_2 has been shown to stimulate SA accumulation in tobacco.

Hydrogen peroxide plays multiple roles in plant stress and defence responses, ranging from wound sealing (via lignin biosynthesis, polysaccharide cross-linking and protein insolubisation in the cell wall); to induction of cell death and possible signalling

functions. Thus both hydrogen peroxide and hydrogen peroxide removing enzymes such as catalase may be closely involved in the regulation or modulation of stress responses in plants.

3.1.5 Chapter summary

The isolation, sequencing and characterisation of a cassava storage root catalase cDNA expressed during post-harvest physiological deterioration is described. Comparative sequence analysis is discussed, and a phylogenetic tree based on an amino acid alignment of 57 plant catalase sequences is presented. The isolated cDNA clone was used to study catalase transcript accumulation during the post-harvest period; and in response to pre-harvest pruning, ethylene and methyl jasmonate. Catalase transcript accumulation was compared in storage roots of cultivars showing contrasting susceptibility to PPD under storage conditions at CIAT, Colombia and at the University of Bath. Tissue printing techniques were used to examine catalase enzyme activity in the cassava storage root, and to compare relative levels of catalase activity in a range of cultivars showing contrasting susceptibility to PPD.

3.2 Isolation, sequencing, and characterisation of a cassava root catalase cDNA

In order to isolate and characterise putative PPD related cassava catalase cDNA clones, a heterologous screening approach was used. A cassava root cDNA library, previously constructed in λ gt10 using total RNA extracted from roots of cultivar NGA1 three days after harvest (Beeching 1997, Han 2000) was screened using a mixture of 3 *Nicotinia plumbaginifola* catalase cDNAs (Willekins *et al.* 1994) as probes.

For probe construction *E.coli* strain DH5α was transformed with plasmids pCAT1, pCAT2 and pCAT3. Plasmid DNA was then isolated by the alkaline lysis method as described in section 2.7.2, and cDNA inserts were excised from the plasmids by restriction digestion. Digests were electrophoresed on a 1% TAE gel and inserts were purified using the Pharmacia Sephaglass method according to the instructions of the manufacturer. Purified inserts (20ng each) were used to prepare an α ^{32}P labelled probe mixture using the Pharmacia "Ready to Go" dCTP labelling kit as described by the manufacturer. Library screening was carried out as described in section 2.7.6. For first round, high density screening duplicate filters (~10^4 plaques per plate) were prepared and hybridisation was carried out overnight at 56°C. Filters were washed to a final wash stringency of 2X SSC at 60°C. After autoradiography, duplicate positive plaques were cored out and used to prepare second round screening plates at lower plaque density (~

400 plaques per plate). Duplicate filters were prepared and screened by hybridisation as previously described. A single duplicate positive from second round screening was cored out into SM buffer and the recombinant phage cDNA insert amplified by PCR using λgt10 forward and reverse primers as described in section 2.7.7. The PCR amplification product was ~1.8kb in size. Following band purification, initial sequencing of the PCR product was carried out using λgt10 forward and reverse primers. Sequence data was submitted to a blastx search using the NCBI database (www.ncbi.nlm.nih.gov) in order to confirm clone identity. Phage DNA was then amplified and isolated using the infection at high multiplicity culture lysate protocol (Sambrook *et al.* 1989) as described in section 2.7.8, and the cDNA insert was excised from the λ arms by *Eco*RI digestion. Electrophoresis revealed that the cDNA comprised an ~1.8 Kb transcript with what was assumed to be a single internal *Eco*RI site (figure 3.2.1). Both large (~1.2kb) and small (~0.6kb) fragments were subcloned into pBluescript KS at the *Eco*RI site. These plasmids were designated MecCAT1a and MecCAT1b (*Manihot esculenta* cDNA encoding catalase) respectively and were sequenced along both strands. For initial sequencing reactions, sequencing was carried out using pUC/M13 forward and reverse primers, for subsequent sequencing reactions primers were designed using the Primer Designer for Windows programme (Scientific and Educational Software). Overlapping sequence data for both strands was compiled using the GELSTART set of programmes within the gcg package (Devereux *et al.* 1984). The sequencing strategy used is shown in figure 3.2.2.

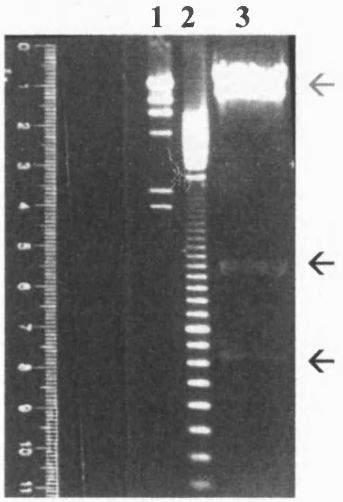


Figure 3.2.1 *Eco*RI digestion of lambda DNA to release the cassava catalase cDNA insert. Lane 1 = marker DNA (wild type lambda *Hind*III digest). Lane 2 = 100bp ladder. Lane 3 = large scale lambda DNA digest. Released catalase cDNA insert fragments are indicated by black arrows. Lambda vector "arms" are indicated by blue arrows.

Once sequence data for both MecCAT1a and MecCAT1b had been compiled, their relative orientation in the original lambda clone could be deduced by the presence of λ adapter sequences in both plasmid sequences as shown in figure 3.2.2. In order to ensure that only a single internal *Eco*RI site was present in the original λ clone, and therefore that no part of the original clone had been "lost" during *Eco*RI digestion, a primer (KC3) was designed within the deduced sequence of MecCAT1a and was used to sequence the original 1.8kb PCR product. When this sequence data was added to the gcg sequencing project, it confirmed the presence of a single *Eco*RI site only, and allowed the overlapping of MecCAT1a and MecCAT1b sequence data to generate the full clone sequence.

The full clone sequence was designated MecCAT1 and has been submitted to the GenBank database under the accession number AF170272. Subsequent analysis of the nucleotide sequence and deduced translation was carried out either "by eye" or using the following programmes- GELSTART, CLUSTALW and FASTA subroutines of telnet gnome GCG package (Devereux *et al.* 1984), Chromas v. 1.43 (McCarthy 1997), GeneDoc (Nicholas and Nicholas 1997), TreeconW (Van de Peer and De Wachter 1994), National Center for Biotechnology Information NCBI (www.ncbi.nlm.nih.gov/), ORF finder (www.ncbi.nlm.nih.gov/gorf/orfig/cgi), PSORT prediction of protein localization sites (www.psort.nibb.ac.jp), PredictProtein (www.embl.heidelberg), SignalP V2 software (http://genome.cbs.dtu.dk/htbin/nph-webface) (Nieilsen *et al.* 1997), ProtParam tool (http://expasy.cbr.nrc.ca/cgi-bin/protparam).

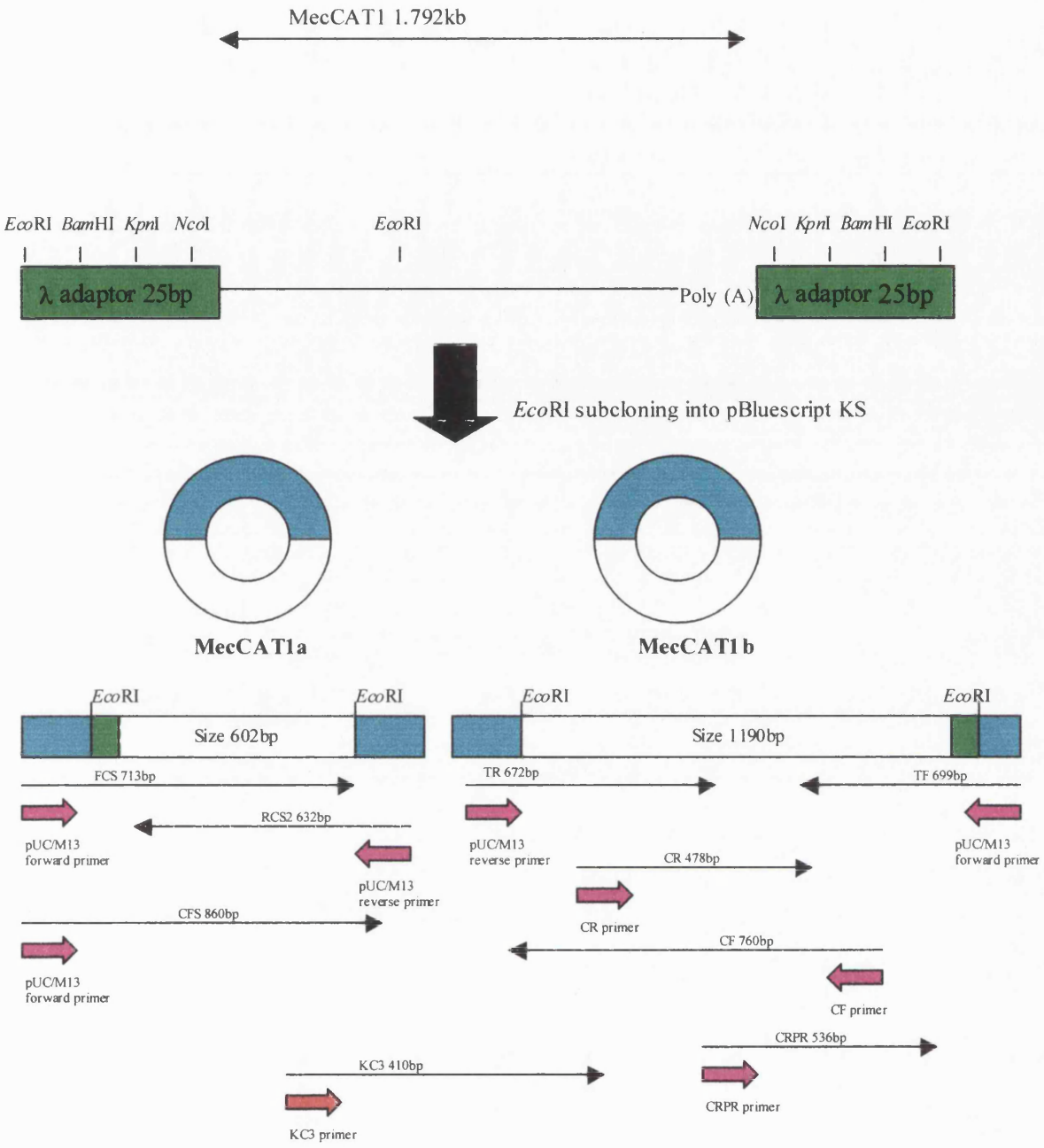


Figure 3.2.2. Diagrammatic representation of the sequencing strategy used for sequencing of MecCAT1. Phage λ *Eco*RI adapter sequences are shown in bright green, plasmid sequences are shown in pale green. Primers are shown in purple (or red) and sequences obtained are indicated by a black arrow above the primer. The relative orientation of sequences was largely deduced by the presence of *Eco*RI adaptor sequences in FCS, CSF and TF; and the presence of an *Eco*RI site (assumed to be a single MecCAT1 internal *Eco*RI site) in RCS2, TR and CF. In order to confirm the overlap, a primer KC3 was designed from the FCS sequence and was used to sequence the ~1.8kb catalase PCR product. The KC3 sequence was included in the gcg sequencing project and confirmed the expected overlap with no sequence data absent in either MecCAT1a or MecCAT1b.

The cDNA clone, designated MecCAT1 represents a cassava catalase transcript of 17.92kb. It encodes a full length predicted protein of 492 amino acids with highest similarity to the (castor bean) *Ricinus communis* Cat2 protein (91% pairwise amino acid similarity). Interestingly the *R.communis Cat2* mRNA is predominantly expressed in roots and in hypocotyls of germinating castor bean seedlings (Suzuki *et al.* 1994). Similarity to the tobacco SABP catalase sequence was 75% pairwise amino acid identity. The MecCAT1 sequence contains a short 5' untranslated region (UTR) with little similarity to other plant catalases cDNAs, although the sequence surrounding the start codon TT**GTCATGG** is similar to the Kozak consensus CCGCC**ATGG** sequence identified as optimal for eukaryotic translation initiation (Kozak 1986). In common with the majority of plant and vertebrate genes the -3 and +4 positions, which are thought to be crucial for fidelity of translation initiation, are occupied by purines (A or G) with G in the +4 position (Joshi *et al.* 1997). The 3' UTR contains 2 exact consensus polyadenylation signals (AATAAA), however these are located at -105bp and -130bp upstream of the poly(A), rather than at the expected –11 to –30bp upstream of the site of poly(A) addition. A similar situation was found for barley *Cat2* where no recognisable polyadenylation signal was found in an appropriate position (Skadsen *et al.* 1995).

The deduced protein has a predicted molecular weight of 57.2 KDa and contains conserved active site residues His 65, His 79, Ser 104 and Asn 138 found in other eukaryotic and prokaryotic catalases; and distal (Pro 331, Arg 353, Tyr 353) and proximal (Val 63, Thr 105, Phe 143) heme binding residues involved in formation of the heme pocket in the mature tetramer (Reid *et al.* 1981, Scandalios *et al.* 1997, Ossowski *et al.* 1993). With regard to localisation, the transcript contains both an internal Ser-Arg-Leu motif located 9 residues from the stop (TGA) codon (consensus Ser - Lys/Arg - His/Leu); as well as a carboxy terminal sequence – Pro-Asn-Ile (consensus Pro - Ser/Thr/Asn - Met/Ile) proposed by Mullen *et al.* (1997), suggesting that the cassava MecCAT1 protein may be localised to glyoxysomes or unspecialised microbodies within the root. The nucleotide sequence and deduced translation of MecCAT1 is shown in figure 3.2.3

3.3 Comparative sequence analysis

Several catalase phylogenetic trees based on nucleic or amino acid sequence data have been published (Klotz *et al.* 1997, Frugoli *et al.* 96, Scandalios *et al.* 1997, Willekins *et al.* 1994, Frugoli *et al.* 1998). The analysis of Klotz *et al.* (1997) included 70 catalase

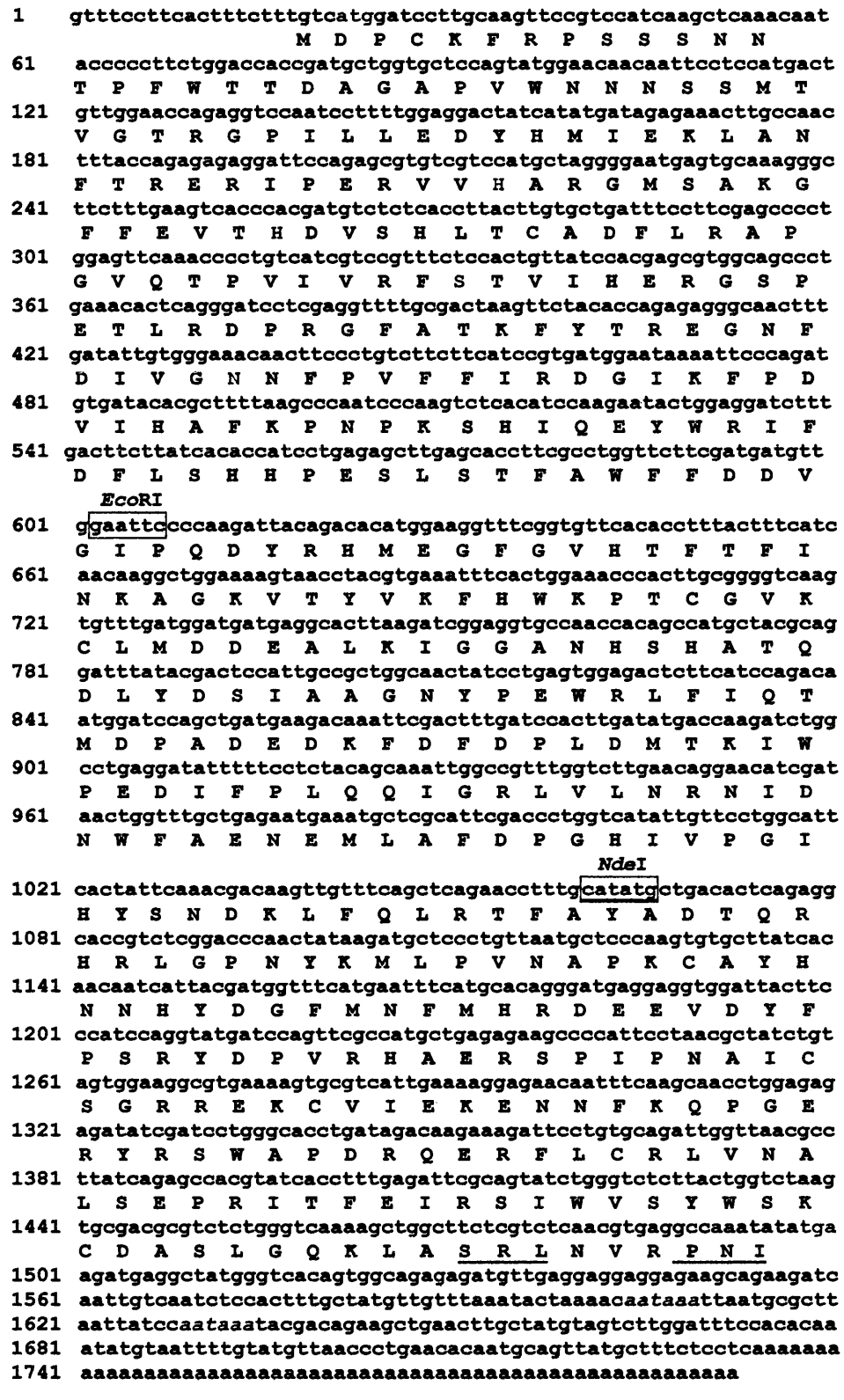


Figure 3.2.3 Sequence and deduced translation of *M.esculenta* MecCAT1. Sequence features referred to in the text are shown in colour. Putative start (ATG) and stop (TGA) codons are shown in red; potential polyadenylation signals (consensus AATAAA) are shown in red italics. Conserved active site residues H65, H79, S104 and N138 are shown in pink. Conserved distal and proximal heme binding residues - V63, T105, F143; and P331, R353 and Y360, are shown in blue and green respectively. Putative peroxisomal targeting motifs are shown in bold and underlined. *Eco*RI and *Nde*I restriction sites used to generate a 459bp putative exon specific probe discussed in section 3.4 are shown boxed.

amino acid sequences of prokaryotic, fungal, plant and animal origin; and divided catalases into 3 major groups, consistent with 2 gene duplication events producing a minimum of 3 catalase gene family members which subsequently evolved in response to environmental demands. Group I of the superfamily includes bacterial group I and the plant catalases; Group 2 includes bacterial group II and fungal group II catalases; and Group III contains bacterial group III, fungal group I and the animal catalases. Plant catalases fall into 3 subgroups as was also found by Guan and Scandalios in an analysis based on 16 sequences (1996).

The amino acid sequences for 57 plant catalases available on the NCBI Genbank database were aligned using the CLUSTALW programme (Thompson *et al.* 1994). A number of partial sequences are also available, however these were not used. Alignments in PHYLIP interleaved format (Felsenstein, 1994) were used to construct an unrooted tree by the neighbour joining method using the Tajima and Nei algorithm (Tajima and Nei, 1984) with the TreeCon 1.2 package (Van de Peer and De Wachter 1994). The dendrogram is shown in figure 3.3.1. The results obtained are consistent with those found by Scandalios *et al.* (Guan and Scandalios 1996, Scandalios *et al.* 1997), Frugoli *et al* (1998) and Klotz *et al.* (1997). The dendrogram presented here contains an additional 41 sequences relative to Scandalios *et al.* (Guan and Scandalios 1996; Scandalios *et al.* 1997), an additional 18 relative to Frugoli *et al.* (1998) and additional 36 plant catalases relative to Klotz *et al.* (1997). Plant catalases fall into 3 subgroups as was found in previous analyses. Group III contains the monocot specific catalases, Group II the dicot catalases and Group I both monocot and dicot catalases. The observed branching pattern is generally congruent with expected phylogenetic relationships. For example, within Group I there is a readily distinguishable legume cluster containing the *Glycine max, Pisum sativum, Pisum vulgaris* and *Vigna radiata* sequences; as well as a Brassica cluster containing *Raphanus sativus, Brassica juncea* and *Arabidopsis* sequences. Monocot sequences (*Zea mays, Oryzae sativa, Triticum aestivum, Zantedeschia aethiopica* and *Hordeum vulgare*) within Group I form a distinct cluster separate from the dicot sequences. Interestingly, the *Chlamydomonas* sequences group with the monocots, as was also found in the analysis of Frugoli *et al.* (1998). Within Group II, a Solanaceae cluster (containing *Lycopersicon, Solanum, Capsicum* and *Nicotiana* species), again supported by high bootstrap values is found; as is a second legume cluster. Cassava MecCAT1 occurs within the Group II catalases forming a small cluster with another euphorbiaciae sequence – *Ricinus communis* (castor bean) Cat2.

The branching pattern may also reflect sorting on a functional as well as phylogenetic basis as suggested by Frugoli *et al.* (1996). For example, of the 3 *C.pepo* catalases (Esaka *et al.* 1997), Cat2 and Cat3 that are down regulated during senescence, group with the Group II catalases; whilst Cat1, which shows up-regulation during senescence, falls in the Group I catalases. Of the 3 *Arabidopsis* catalases (McClung 1997), Cat1 and Cat2 are light inducible and are found in Group I, whilst the light down-regulated Cat 3 occurs in Group II. Cassava MecCAT1 (which is known to be expressed at high levels in cassava roots, with little expression in leaves) groups with *R. communis* Cat2, which likewise is expressed at high levels in roots (Suzuki *et al.* 1994). A second *R. communis* catalase - Cat1 – that is highly expressed in endosperms and cotyledons but not roots, groups separately with the Group I catalases. A detailed survey of expression patterns of plant catalases would be of interest in order to elucidate possible grouping on the basis of function.

Part of the alignment used for tree construction, showing an alignment of cassava MecCAT1 with 4 other plant catalase sequences, is shown in figure 3.3.2. (The full alignment containing all 57 sequences is shown in appendix A). A high degree of conservation amongst plant catalases is evident, as may be seen by the colour blocking of conserved residues between both monocot and dicot species. For the purposes of the alignment shown in figure 3.3.2, sequences for which experimental data on sub-cellular localisation were available were chosen. *Zea mays* Cat3 has been shown to co-isolate exclusively with mitochondria in cell fractionation studies, whilst *Zea mays* Cat1 and Cat2 are targeted to microbodies (Scandalios 1983, Scandalios *et al.* 1997). Esaka *et al.* (1997) have shown in immuno-cytochemical studies using a Cat1-specific anti-peptide antibody that *C.pepo* Cat1 catalase is located in glyoxisomes. With regard to targeting signals, it is interesting to note that all of the experimentally-verified microbody-targeted protein sequences (*Z.mays* Cat1 and Cat2, *C.pepo* Cat1), as well as the cassava MecCAT1 deduced protein, contain a carboxy terminal tripeptide motif in agreement with that proposed by Mullen *et al.* (1997) as the plant catalase microbody targeting motif (consensus Pro – Ser/Thr/Asn – Met/Ile). The mitochondria targeted *Z.mays* Cat1 in contrast, contains the non consensus motif Ala – Asn – Met at this position.

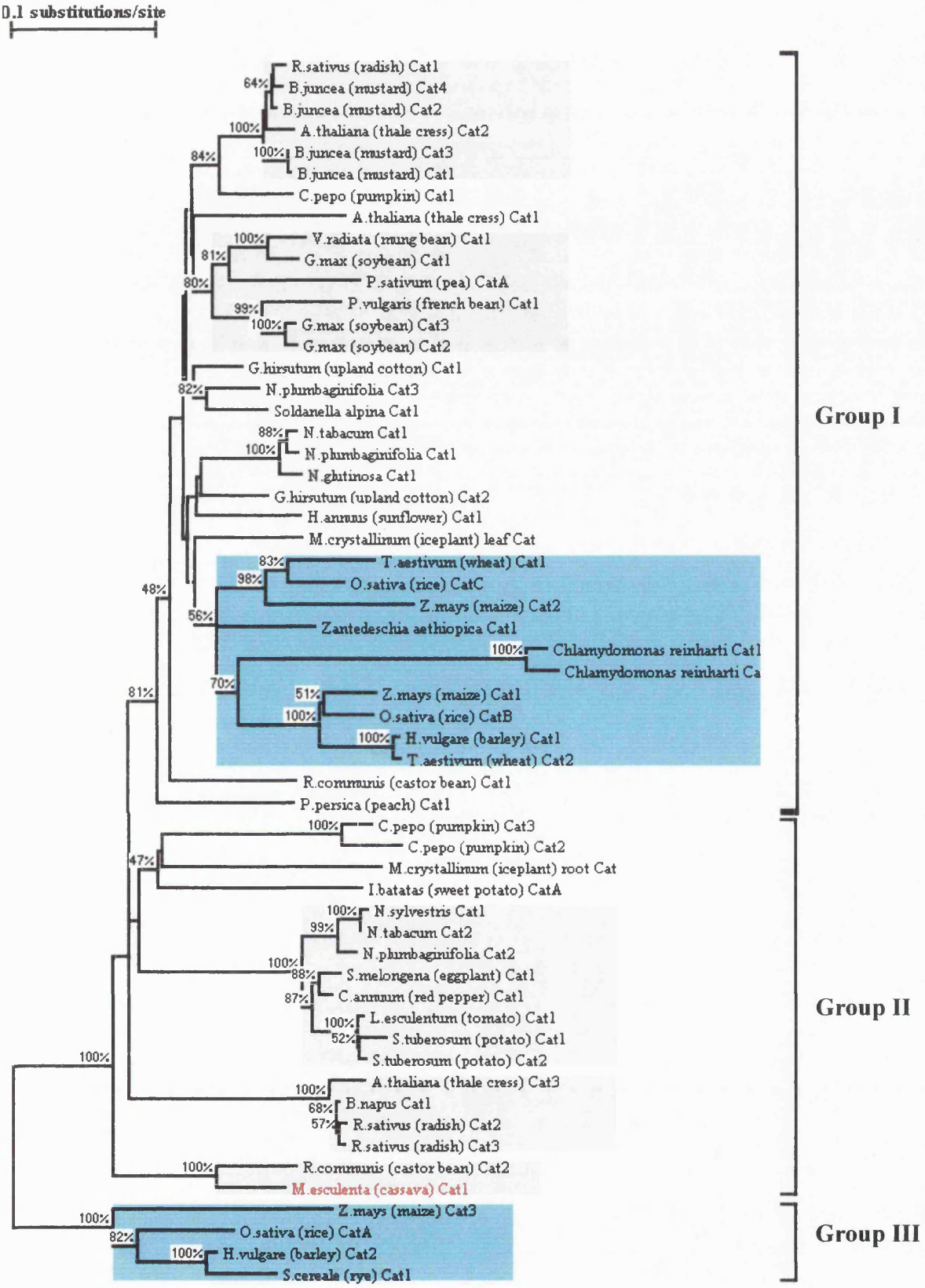


Figure 3.3.1 Unrooted tree constructed from 57 plant catalase amino acid sequences using the Tajima and Nei (1984) algorithm. Branch lengths reflect the number of amino acid changes per 1000 residues. Bootstrap re-sampling values above 50% are shown at the nodes. Legume, Solanaceae, Brassicae and Euphorbiaciae clusters referred to in the text are shown in grey. Monocot clusters are shown in blue. Cassava catalase MecCAT1 is shown in red.

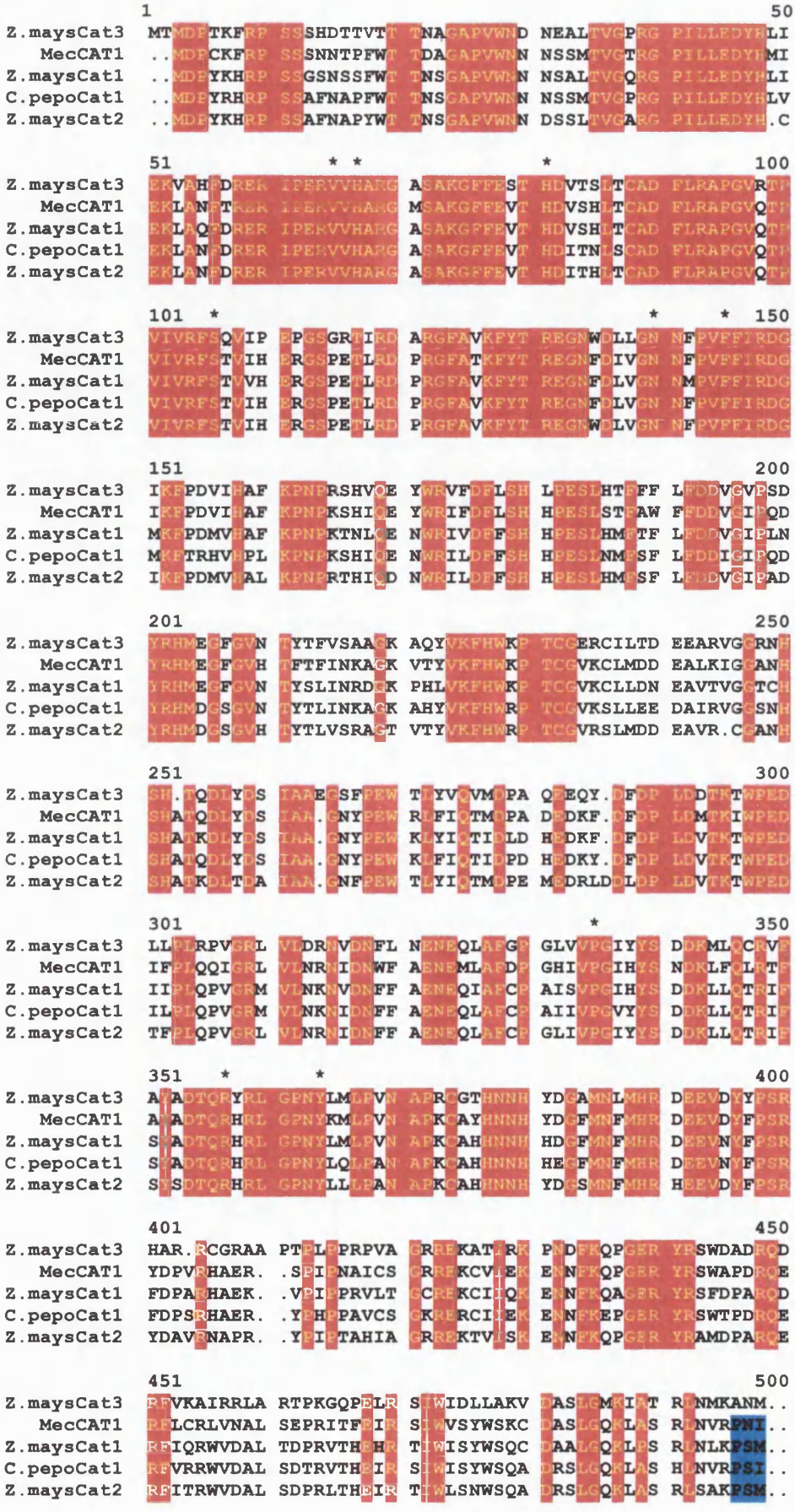


Figure 3.3.2 Amino acid alignment of cassava MecCAT1 with other plant catalases. Residues conserved in all 5 proteins are shown in red/yellow colour blocking. Conserved active site and heme binding residues are indicated by asterisks. Putative microbody targeting motifs are shown in blue.

3.4 Catalase gene organisation in cassava

In order to determine catalase gene number in the cassava genome, Southern blots of genomic DNA (cultivar NGA1) digested with the restriction enzyme *Eco*RV, *Xba*I and *Hind*III were prepared as described in section 2.7.9. Blots were probed initially with the 1.19kb *Eco*RI fragment of the MecCAT1b cDNA (figure 3.2.2). Hybridisation was carried out overnight at 60°C, and the membrane was washed to a final wash stringency of 1X SSC, 0.1% SDS at 60°C. Results are shown in figure 3.4.1. For the MecCAT1b probe (of size 1190bp) the minimum percentage homology required to allow stable probe-target hybridisation under these stringency conditions was 81.1%, as calculated using the equation $Tm°C = 81.5°C + 16.6\log [Na^+] + 0.41(\%GC) - (600/1)$ as described in section 2.7.9 and assuming a GC content of 50%. Restriction analysis of the sequence using the Clone Manager for Windows (v.4) programme (Scientific and Educational Software, 1995) indicated that this region of the cDNA contained a single restriction site for *Eco*RV, and no sites for the restriction enzymes *Xba*I and *Hind*III. Since 5 hybridising bands were detected in the *Eco*RV lane, with 4 in the *Xba*I and *Hind*III lanes (figure 3.4.1 panel A), these data would be consistent with the presence of a small catalase gene family in cassava.

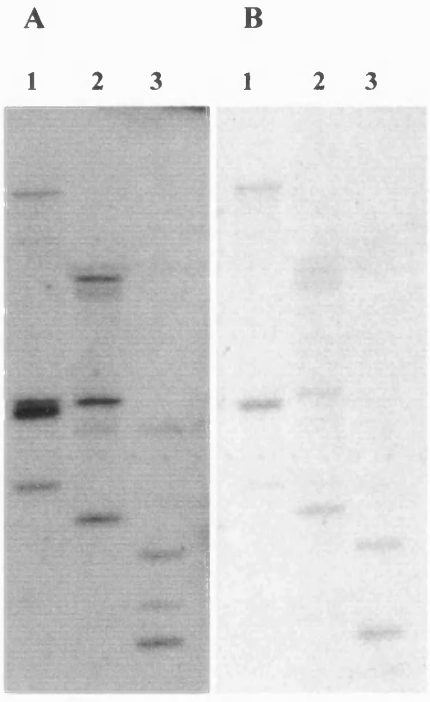


Figure 3.4.1 Southern blot analysis of cassava nuclear gene organisation. Genomic DNA (10μg per lane) was digested with the restriction enzymes *Eco*RV, *Xba*I, and *Hind*III. The same southern blot was probed initially with a 1.19kb *Eco*RI fragment of MecCAT1 (panel A), after autoradiography the probe was stripped from the membrane and the blot was reprobed with a 459bp *Eco*RI/*Nde*I putative exon specific probe (panel B). Lane 1 = *Eco*RV digest, Lane 2 = *Xba*I digest, Lane 3 = *Hind*III digest.

A limitation in the use of cDNA probes to estimate the number of related genes by Southern blotting, is the possible occurrence of restriction sites within intron sequences. Dicot catalase genes analysed to date contain 6 to 7 introns (Iwamoto *et al.*, 1998; Scandalios *et al.*, 1997). In order to exclude multiple hybridising bands due to restriction sites within the introns of the cassava MecCAT1 gene, a putative exon specific probe was designed. In a recent analysis of the exon-intron structure of the 12 plant catalase genomic sequences available, it was shown that intron position and the size of the exons, with the exception of the first and last, were conserved among plant catalases. In addition, although the sizes of the first and last exons were variable, the lengths of amino acid coding regions within these exons was conserved, with the difference in size being conferred by variable lengths of 5' and 3' un-translated regions (Iwamoto *et al.* 1998). In 5 of the 6 dicot catalase genomic sequences available, exon 4 is 777 bp in length; in the soybean Cat gene intron 3 has been lost resulting in a 1055 bp exon 3, which comprises a fusion of exons 3 and 4 of the other dicots (figure 3.4.2) Thus a comparison of the cassava MecCat1 cDNA and deduced translation, with the dicot exon 4 sequences was used to design a 459bp *Eco*RI / *Nde*I fragment of MecCAT1 which should correspond to most of the coding region of exon 4 conserved in other dicot catalase genes. (Restriction sites used to generate this probe are shown in figure 3.2.3). Blots were stripped and re-probed with the putative exon specific probe under the same conditions as previously (figure 3.3.1 panel B). The sequence of the 459bp putative exon specific probe does not contain restriction sites for *Eco*RV, *Xba*I and *Hind*III. Thus, since 3 hybridising bands remain in the *Eco*RV and *Xba*I lanes, these data suggest that there are at least 2 or 3 catalase genes in the cassava genome. These data would be consistent with the occurrence of 3 catalase gene family members in the well characterized plant systems tobacco, maize and *Arabidopsis*

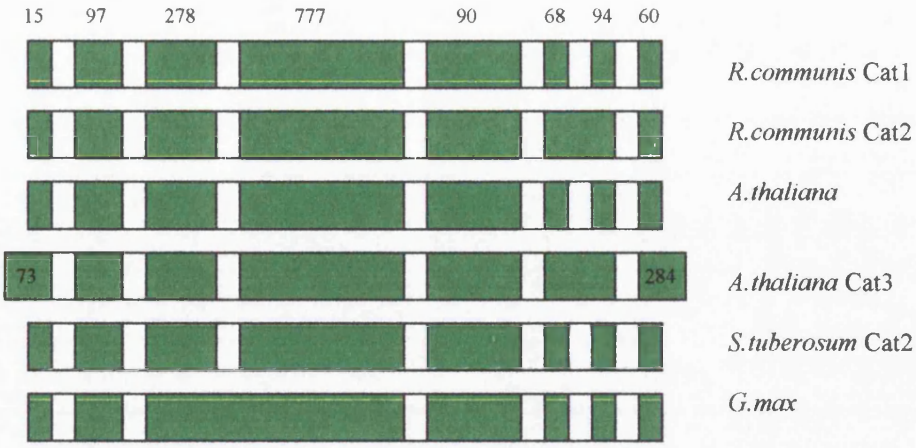


Figure 3.4.2 Intron/exon structure of dicot catalase genes (after Iwamoto *et al.* 1998). Exon sequences are shown in green. The size of the exons are indicated in base pairs.

3.5 MecCAT1 transcript accumulation during post-harvest storage

To examine MecCAT1 transcript expression, total RNA was extracted from storage roots and leaves of cultivar MNGA 2 according to a modification of the method of Chang *et al.* (1993) (S.Bohl personal communication) as described in section 2.7.10. Total RNA (10µg per lane) was electrophoresed on 1.5% agarose gel containing formaldehyde and blotted onto nylon membrane (Hybond N plus, Amersham) according to standard procedures (Sambrook *et al.* 1987). Hybridisations were carried out at 65°C overnight and membranes were washed to a final wash stringency of 0.2X SSC, 0.2 % SDS for 20 minutes at 65°C; and were then visualised by autoradiography at -70°C.

Cassava storage roots for these experiments were wax dipped immediately after harvest and air freighted from CIAT, Colombia. On arrival, post-harvest physiological deterioration was induced by removal of the proximal and distal ends of the root and cutting of 2 "V" shaped incisions through the epidermis along the length of the root. The root ends were covered with parafilm and roots were stored at ambient temperature on a bench top. Samples were removed for RNA extraction over a time course of 8 days. Under these storage conditions, symptoms of vascular streaking appeared in the root 4 days after injury. Leaf material was obtained from greenhouse grown plants at the University of Bath. As a control for equal loading, the gel was viewed under UV light and documented by photography.

Results are shown in figure 3.5.1. The MecCAT1 transcript showed up-regulation during the post-harvest storage period, and was predominantly expressed in roots with lower levels of expression detected in leaves.

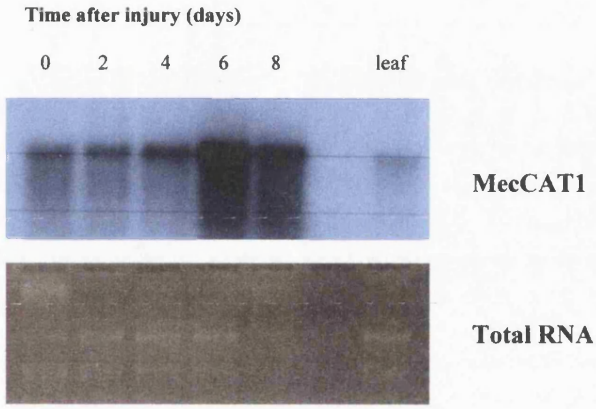


Figure 3.5.1 mRNA accumulation of MecCAT1 in storage root and leaves of cultivar MNGA2. As a control for equal loading the gel was documented under UV light (lower panel).

3.6 Effect of pruning treatment, ethylene and jasmonic acid on MecCAT1 transcript accumulation

Transcript accumulation of MecCAT1 following pre-harvest pruning, and in response to treatment with ethephon and methyl jasmonate was examined by northern blotting as described in section 2.7.10. Treatments were carried out as described in section 2.7.10.8. Total RNA was extracted from storage roots of cultivar MCOL 22 at CIAT, Colombia. Root samples were taken on the day of harvest. Attempts were made to extract RNA from root samples treated with the plant signalling molecule salicylic acid, which has been implicated in induction of defence related genes in several plant systems (Murphy *et al.* 1999, Scott *et al.* 1999, Yu *et al.* 1997). However, good quality RNA was not obtained after two attempts from either control or treated samples and this experiment was not continued.

For the pre-harvest pruning treatment, roots were obtained from plants which had been pruned by removal of the stem and all leaves approximately 30cm from the ground 2 weeks prior to harvest. Control RNA samples were prepared from similar non pruned plants harvested at the same time. For the ethylene treatment, root slices were incubated in the ethylene generating compound ethephon (Sigma) (0.02% in sterile water) for 24 hours in the dark. Control slices were incubated in water alone. For the methyl jasmonate treatment, root slices were incubated for 24 hours in the dark in methyl

jasmonate (Sigma) (500μM in 0.1% ethanol). As noted by Plumbley *et al.* (1981) accelerated deterioration was observed in root slices that had been treated with ethylene. Hybridisation with MecCAT1b as a probe showed a transcript of approximately 2kb. The transcript was strongly induced by pre-harvest pruning, and showed up-regulation in response to ethephon treatment. Root treatment with methyl jasmonate induced a slight down-regulation of MecCAT1 transcript accumulation (figure 3.6.1 Panels A, B and C respectively).

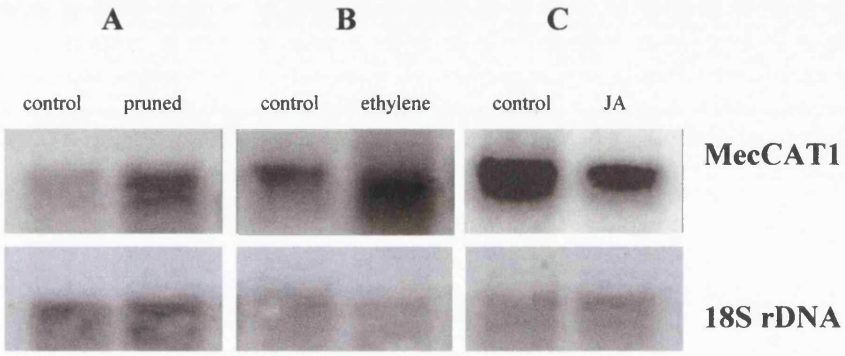


Figure 3.6.1 mRNA expression of cassava catalase MecCAT1 in response to different treatments. For all treatment panels, control samples are shown on the left, experimental treatment samples are shown on the right. Panel A = Pre-harvest pruning treatment. Panel B = Ethephon treatment. Panel C = Jasmonic acid treatment.

3.7 Tissue localisation of catalase enzyme activity

For the detection and localisation of catalase enzyme activity in cassava storage roots, a modified tissue printing protocol based on the starch gel method of Manchenko (1994) was used, as described in section 2.7.14. Tissue prints were made onto pieces of starch impregnated nitrocellulose membrane (Hybond C, Amersham) and immersed in 60mM sodium thiosulphate: 3% hydrogen peroxide solution (3: 7). Bubbles of molecular oxygen (O_2) produced by the action of catalase on hydrogen peroxide were observed in the area of the print. The membrane was then transferred to 90mM potassium iodide, 0.5% glacial acetic acid, placed on blotting paper and documented by photography. The hydrogen peroxide saturates the nitrocellulose membrane, except in areas where it is broken down enzymatically. On transfer of the membrane to the potassium iodide solution, the iodide is oxidised by hydrogen peroxide to iodine and forms a chromatophore with starch except in areas of catalase activity where the hydrogen peroxide has been removed. The method is thus a negative stain - areas of catalase activity are indicated by clear areas on a dark background. Thiosulphate is incorporated

into the staining solution and is inactivated by hydrogen peroxide, except in areas of catalase activity. It serves to reduce any iodine which may diffuse into the clear areas to iodide. Control reactions carried out on heat treated tissue show little or no development of clear areas. As a control for even protein transfer, tissue prints on nitrocellulose paper were stained to detect total protein using Coomassie blue, as described in section 2.7.14.4. An example of tissue print detection of catalase activity in a cassava storage root transverse section is shown below (figure 3.7.1). Catalase activity, as indicated by clear areas on a dark background, was present throughout the root parenchyma, with little activity commonly observed in the cortex.

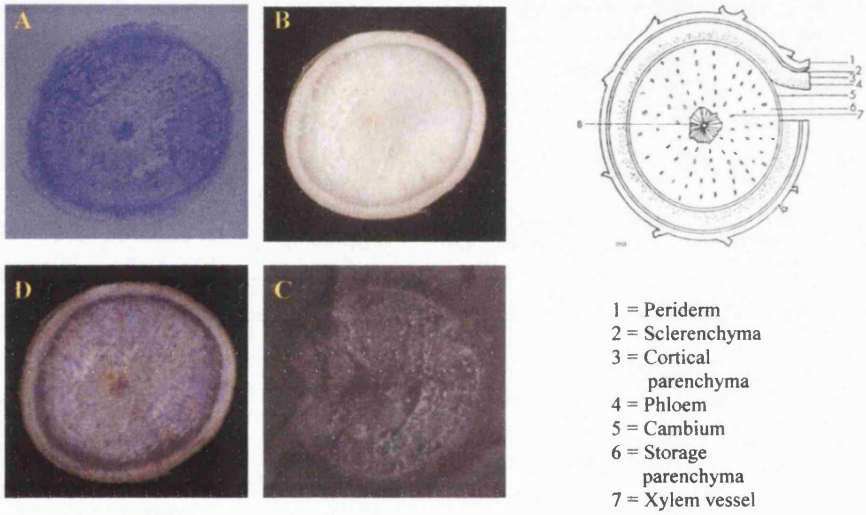


Figure 3.7.1 Tissue print detection and localisation of catalase activity in a cassava storage root of cultivar MCOL22 immediately after harvest. Panel A = Tissue print detection of total protein (Coomassie blue). Panel B = Transverse root slice used for tissue printing. Panel C = Tissue print detection of catalase activity. Panel D = Overlay of images from panels A and B. A schematic representation of the tissues of the cassava storage root (after Hunt *et al.* 1977) is shown on the right.

3.8 Comparative catalase expression in different cultivars under storage conditions at the University of Bath.

MecCAT1 transcript accumulation and overall catalase enzyme activity during the post harvest period were compared for a range of cultivars showing differing susceptibility to PPD. For northern blotting experiments, roots were obtained as air freighted material from CIAT, Colombia, and PPD was induced as previously described in section 3.5. Total RNA was extracted from storage roots of cultivars MCOL 22 and MNGA 2 over an 8 day time course. Cultivar MCOL 22 has been characterised as showing high

susceptibility to PPD, whilst MNGA 2 has been characterised as showing intermediate to low susceptibility. For these experiments, visible symptoms of PPD occurred in the highly susceptible cultivar MCOL 22 by the 2nd day after wounding of the root. In the less susceptible cultivar MNGA 2, slight vascular streaking was observed on day 4, and remained less pronounced than in MCOL 22 at day 8. Northern blotting and hybridisation were carried out as described in section 2.7.10. Samples from both cultivars were run on the same gel in order to allow comparison of hybridisation intensity. Results are shown in figure 3.8.1 Hybridisation with MecCAT1b showed a transcript of approximately 2kb. The transcript was induced during the post harvest storage period in roots of both cultivars, however, somewhat higher levels of MecCAT1 transcript were detected in the less susceptible cultivar MNGA 2.

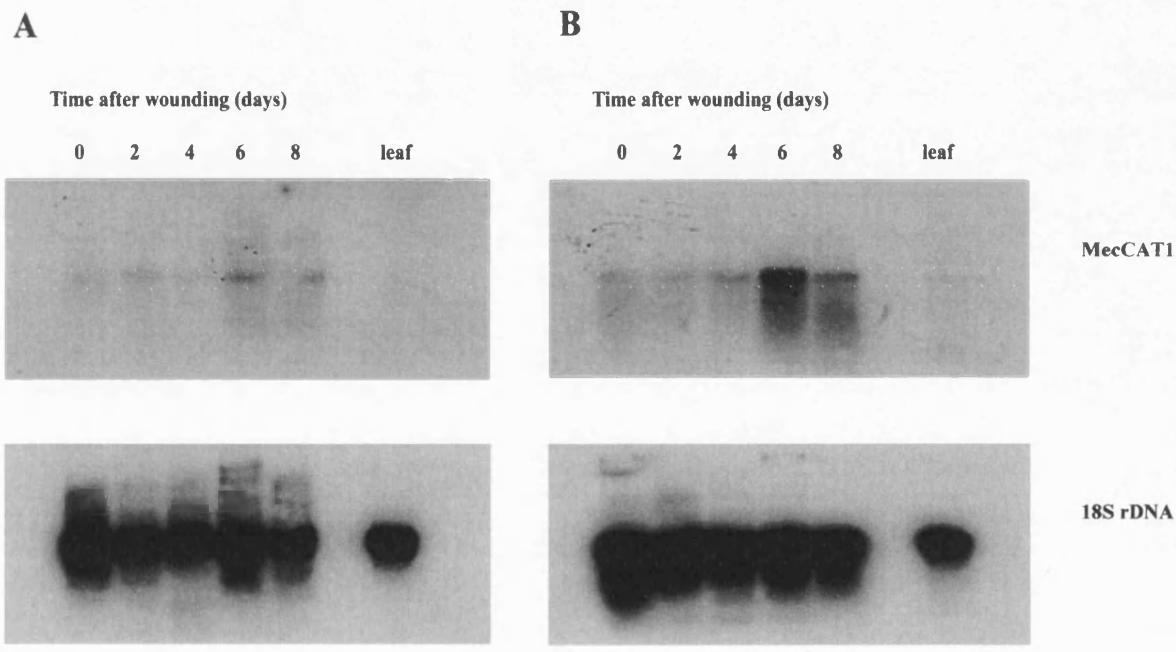


Figure 3.8.1 MecCAT1 transcript accumulation in storage roots and leaves of MCOL 22 (high PPD susceptibility), and MNGA 2 (intermediate PPD susceptibility). Panel A = MCOL 22. Panel B = MNGA2. As a control for equal loading the same blot was stripped and re-hybridised with an 18S rDNA probe (lower panels).

Tissue print localisation of catalase enzyme activity, for the same root samples as were used for northern blotting above, are shown in figure 7.8.2. Higher levels of catalase activity were again observed for the less susceptible cultivar MNGA 2.

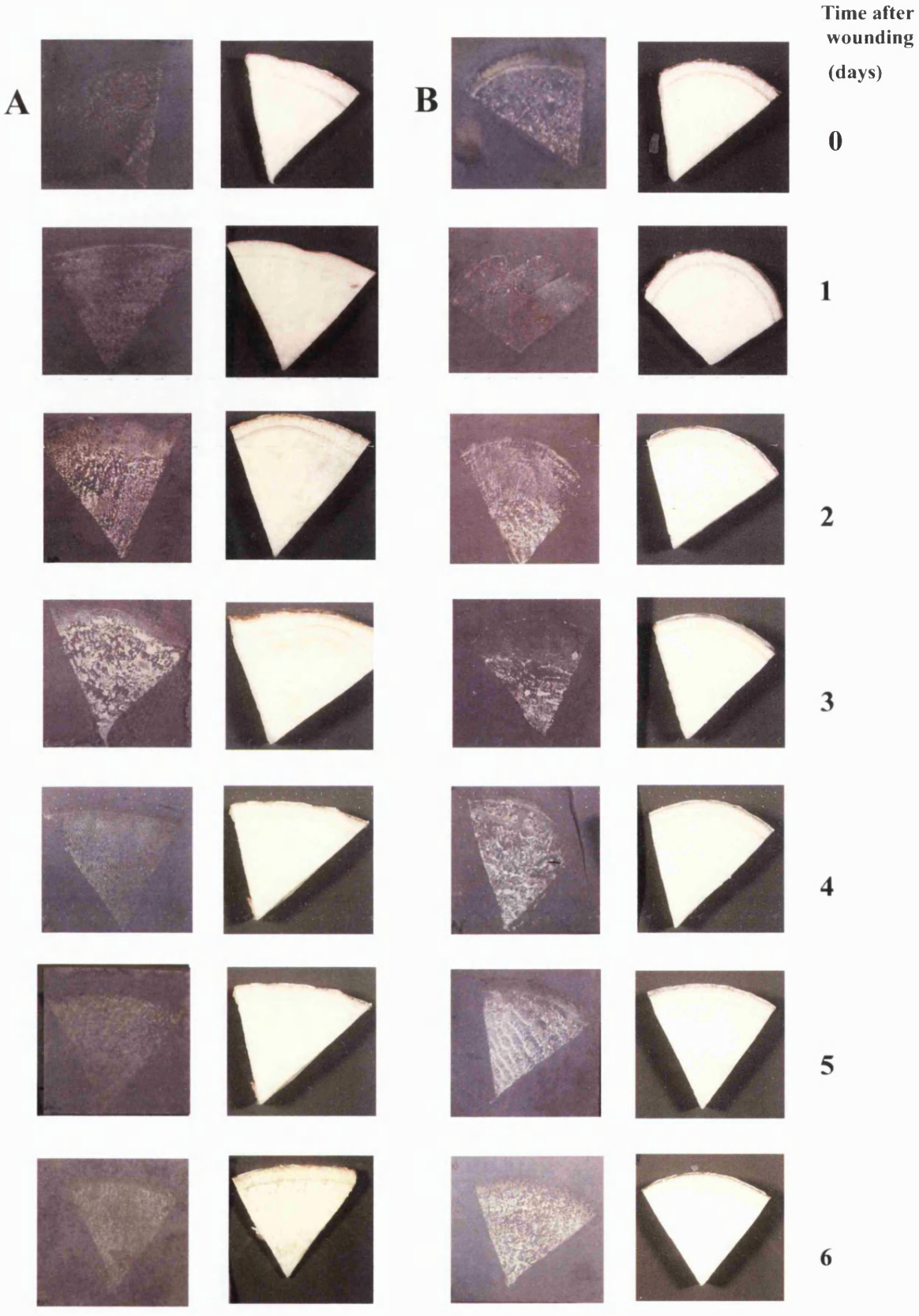


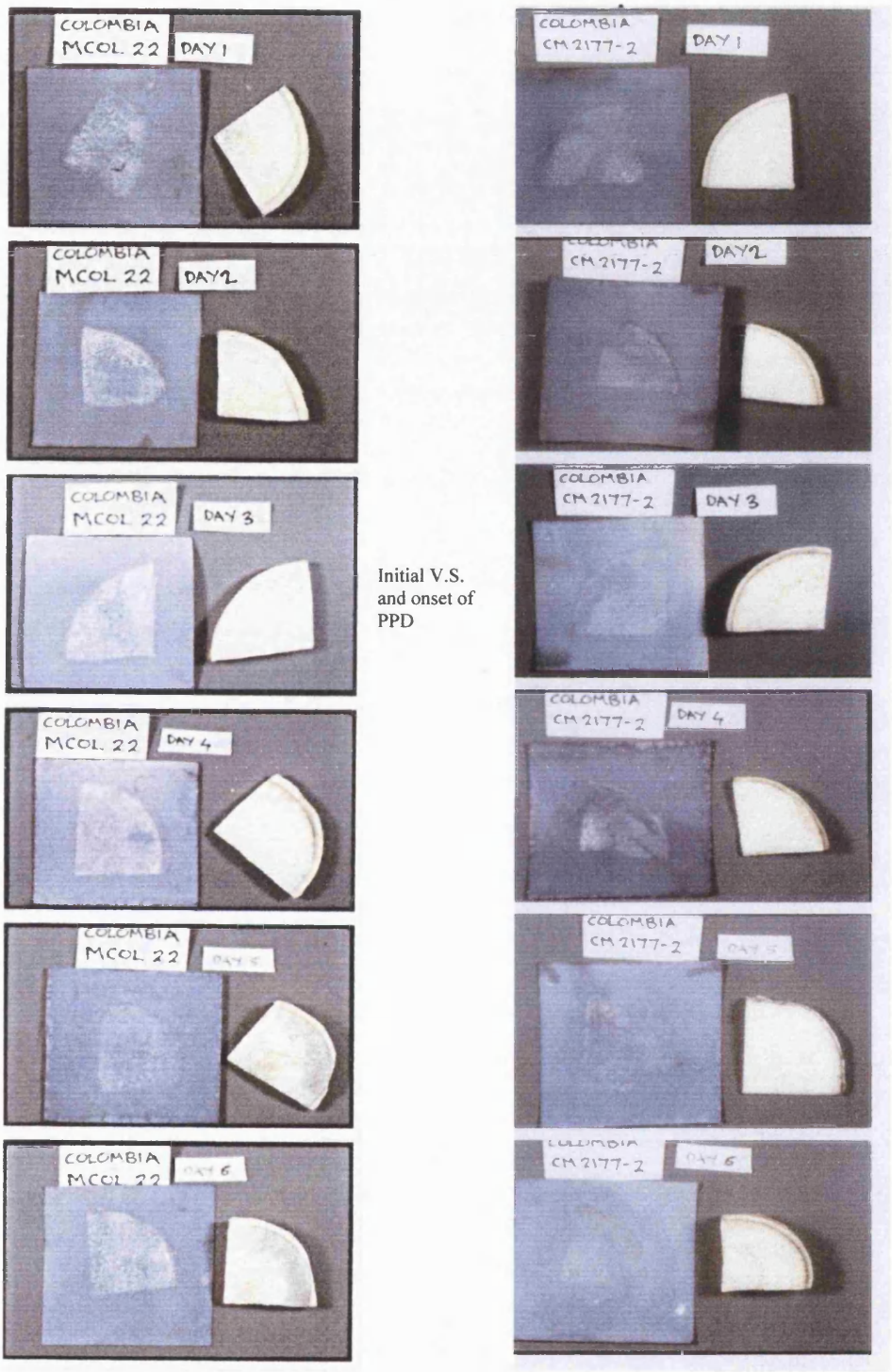
Figure 3.8.2 Tissue print detection and localisation of catalase activity in cassava storage roots. Panel A = cultivar MCOL 22 (highly susceptible). Panel B = cultivar MNGA 2 (less susceptible). For each panel tissue prints are shown on the left, transverse tissue slices from which the prints were prepared are shown on the right.

Although MecCAT1 transcript levels in the susceptible cultivar, MCOL22, were highest at day 6 (figure 3.8.1 panel A), catalase activity as detected in the tissue printing assay had already peaked and was decreasing by this time point (figure 3.8.2 panel A). These data suggest that regulation of activity may be occurring at the level of translation and/ or protein inactivation. Such regulation of catalase activity at the protein translation and/or inactivation level has been reported, for example, in sweet potato roots (Sakajo *et al.* 1987) and pumpkin cotyledons (Esaka *et al.* 1997). In senescing pumpkin cotyledons, cat1 catalase transcript levels increased although the amount of immuno-reactive catalase gradually decreased (Esaka *et al.* 1997). Several mechanisms may be proposed which could account for catalase inactivation, including binding of salicylic acid, free radical attack and enzyme crosslinking by phenolics such as scopoletin (Sanchez Casas and Klessig 1994, Lledias *et al.* 1998, Petit-Paly *et al.* 1999). It is tempting to speculate that phenolic compounds such as catechins, scopolin, scopoletin, esculin and esculetin, which are produced during physiological deterioration of cassava storage roots (Uritani *et al.* 1983, Buschmann *et al.* 2000a), might play a role in catalase inactivation.

Similar comparative tissue print localisation experiments for cultivars MCOL 22 and CMC 21772 carried out in Bath using air-freighted roots from CIAT, Colombia are shown in figures 3.8.3 and 3.8.4. Cultivar MCOL 22 has been characterised as showing high susceptibility to PPD. Cultivar CMC 21772 has been characterised as showing variable susceptibility. Under Bath storage conditions, storage roots of CMC 21772 consistently showed very high susceptibility to PPD, with symptoms of vascular streaking occurring earlier than in MCOL 22.

In order to prevent or minimise initiation of post-harvest physiological deterioration (PPD) in transit, roots were wax dipped and shipped immediately after harvest. On arrival post-harvest physiological deterioration was induced by wounding of the storage roots by removal of the proximal and distal ends and cutting a small "V" shaped incision along the length of the root to remove the epidermis and the wax coating. Root ends were covered in parafilm and the roots were stored at ambient temperature on a lab bench. Samples were taken from the distal end at daily intervals over a time course of 6 or 7 days.

Tissue printing and documentation were carried out as previously described. Results are shown in figures 3.8.3 and 3.8.4. A similar trend in catalase activity is again observed, with lower relative levels of activity detected in the more rapidly deteriorating cultivar, particularly at the later time points after injury.



Control: Tissue slice heat treated in boiling water for 5 minutes.

Figure 3.8.3. Tissue print detection and localisation of catalase activity in storage roots of cassava cultivars showing different rates of PPD. In the more rapidly deteriorating cultivar CMC 21772 vascular streaking (V.S.) occurred 1 day after injury of the root. In cultivar MCOL 22 V.S. occurred 2 days after injury of the root. An example of a control reaction is shown inset on the left.

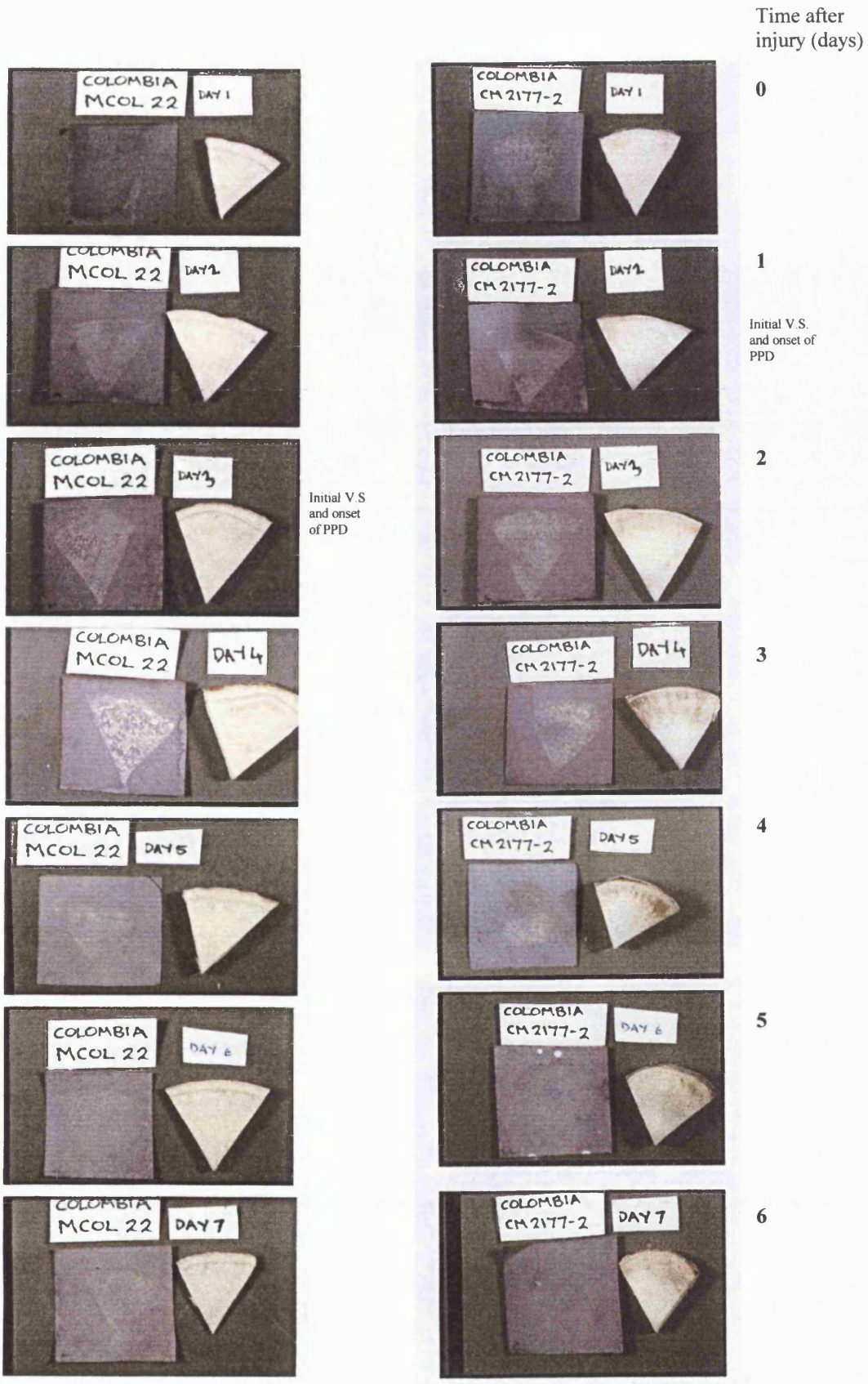


Figure 3.8.4. Tissue print detection and localisation of catalase activity in storage roots of cassava cultivars showing different rates of PPD. In the more rapidly deteriorating cultivar CMC 21772 vascular streaking (V.S.) occurred 1 day after injury of the root. In cultivar MCOL 22 V.S. occurred 2 days after injury of the root.

3.9 Comparative catalase expression in different cultivars under field storage conditions at CIAT, Colombia

Catalase transcript accumulation for MecCAT1 and overall catalase enzyme activity during the post harvest period under field storage conditions were compared in a range of cultivars showing differing susceptibility to PPD. Cassava storage roots for these experiments were freshly harvested from the field and were then injured by removal of the proximal and distal ends of the roots and cutting of 2 "V" shaped incisions through the epidermis along the length of the root. The root ends were covered with parafilm and roots were stored in an open air shed. For northern blotting experiments, total RNA was extracted from storage roots over a 5 day time course. Cultivars used were MCOL 22, MNGA 2, MDOM 5 and CM 21772. These cultivars have been characterised as showing high, intermediate, low and variable susceptibility to PPD. Under the storage conditions used here, visible symptoms of PPD occurred in storage roots of all cultivars within 24 hours after harvest and the PPD response progressed rapidly. Relative rates of deterioration in roots used for RNA extractions were MCOL 22 > MNGA 2 ≥ MDOM 5 whilst CM 21772 showed less pronounced deterioration. Northern blotting and hybridisation was carried out as described in section 2.7.10. Results are shown in figure 3.9.1. Hybridisation with MecCAT1b again showed a transcript of approximately 2kb, with levels of transcript accumulation somewhat higher in the less susceptible cultivars MDOM 5 and CMC 21772.

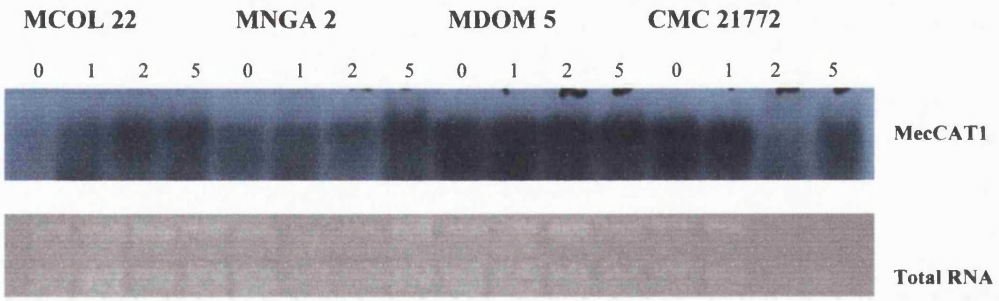


Figure 3.9.1 MecCAT1 transcript accumulation in cassava storage roots over a 5 day storage period. As a control for equal loading the gel was viewed under UV light and documented by photography (lower panel).

In order to compare relative catalase levels at the level of enzyme activity, tissue printing experiments as previously described in section 3.8 were carried out at CIAT, Colombia using roots stored under field conditions as described above. Results are shown in figure 3.9.2. Relative rates of deterioration for the roots used in this experiment were MCOL 22 > MNGA 2 ≈ MDOM 5.

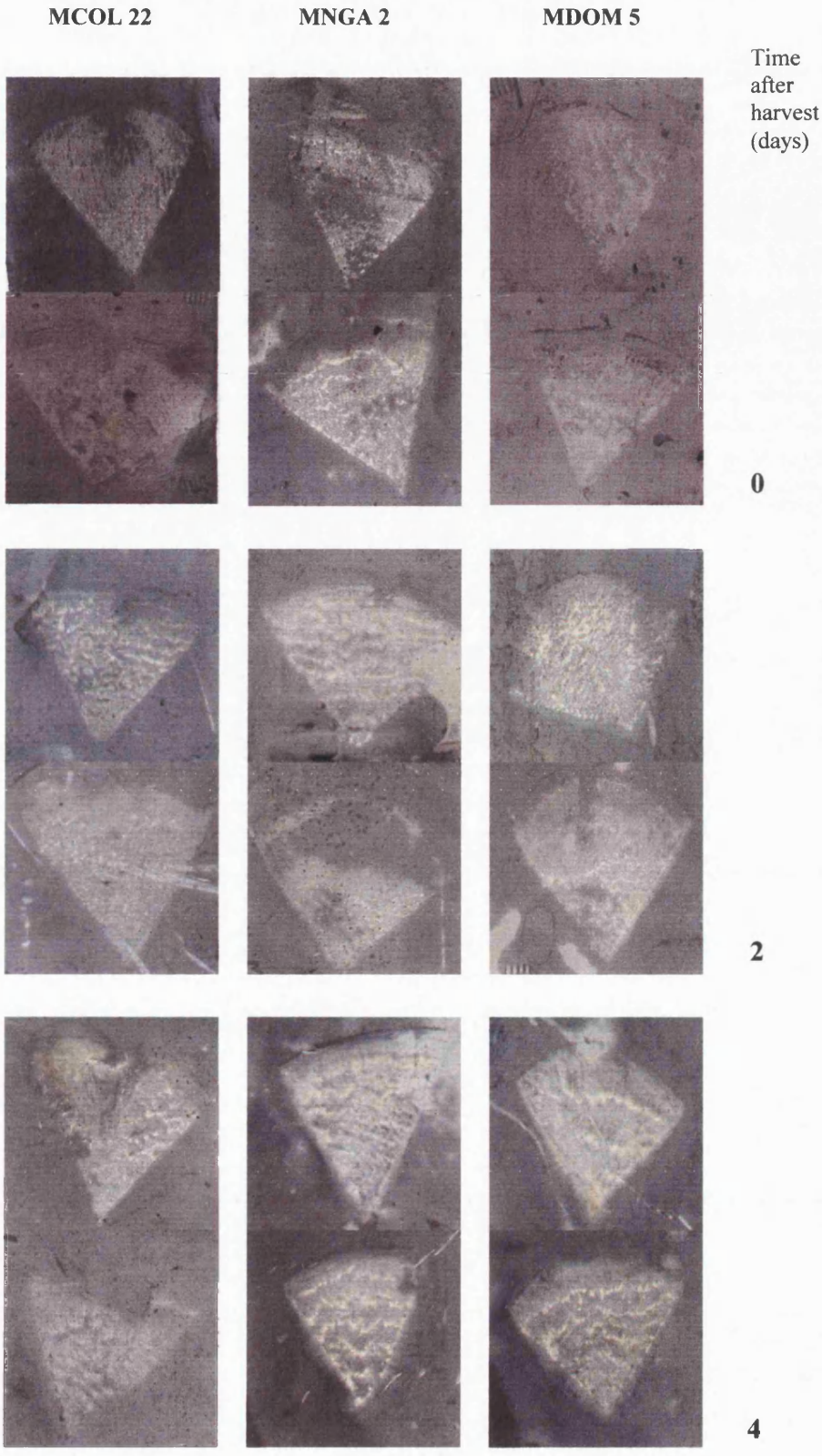


Figure 3.9.2. Catalase activity in cassava storage roots during the post harvest period – experiments carried out at CIAT, Colombia. Catalase activity is indicated by clear areas on a dark background. For each cultivar, transverse root slices were prepared from 2 separate roots. Cultivar MCOL 22 = highly susceptible to PPD. Cultivar MNGA = intermediate susceptibility. Cultivar MDOM 5 = low susceptibility.

3.1.10 Conclusions and discussion

Results presented here describe the isolation and characterisation of a cassava catalase cDNA clone designated MecCAT1. The sequence and deduced translation of MecCAT1 has been lodged with the Genbank database under the accession number AF170272, and the clones have been transferred to CIAT for inclusion on the cassava genetic map. The isolated cDNA is 1792bp in size and encodes a full length predicted protein of 492 amino acid residues. The deduced protein showed highest similarity to *R..communis* (castor bean) Cat2 (91% amino acid identity), and contained a conserved carboxy-terminal motif (Pro-Asn-Ile), in agreement with the proposed microbody targeting signal of plant catalases (Mullen *et al.* 1997). These data suggest that the protein encoded by MecCAT1 may be localised to glyoxysomes within the cassava storage root. Sequence analysis of the predicted protein indicated a molecular weight of 57.2 KDa.

A plant catalase phylogenetic tree based on an amino acid alignment of 57 sequences was broadly in agreement with previously published trees (Guan and Scandalios 1996, Klotz *et al.* 1997, Scandalios *et al.* 1997, Frugoli *et al.* 1998) and contained a number of additional sequences. The cassava MecCAT1 sequence grouped within the plant Group II catalases, forming a small clade with the *R.communis* Cat 2 sequence from a related member of the Euphorbiaceae. As noted by Frugoli *et al.* (1996), the tree shows sorting on a functional as well as phylogenetic basis.

Southern blotting experiments indicated that MecCAT1 formed part of a small gene family in cassava. Southern blot experiments using a putative exon specific probe suggest that the cassava genome contains at least 2, possibly 3 gene family members.

Northern blotting experiments indicated that the MecCAT1 transcript is up-regulated during post-harvest storage and is expressed predominantly in the storage root, with lower levels of expression in leaves.

The transcript was strongly up-regulated in response to pre-harvest pruning, and up-regulated by ethylene treatment. Several studies have indicated that pre-harvest pruning 1 to 2 weeks before harvest can reduce the susceptibility of cassava storage roots to PPD (Tanaka *et al.* 1983, Tanaka *et al.* 1984, Data *et al.* 1984, Kato *et al.* 1991). In this context, it is tempting to speculate that pre-harvest pruning could induce systemic up-regulation of catalase activity, which may play a role in the reduced susceptibility of roots from pruned plants. Ethylene is produced in cassava storage roots in response to injury after a short lag of around 6 hours (Hirose 1986). In other plant systems it has been proposed to be involved in the transmission of the wound stimulus, resulting in

the expression of wound or defence related genes, and is also associated with fruit ripening, floral senescence and abscission.

The apparent slight down-regulation of the transcript to methyl jasmonate is intriguing. In other plant systems JA and methyl jasmonate have been proposed to act as lipid derived signalling molecules resulting in the activation of defence related genes in response to wounding, elicitor treatment and pathogen attack. (Schaller and Ryan 1995, Alami *et al.* 1999), and in the promotion of leaf senescence (Del Rio *et al.* 1998). Jasmonates are derived via the octadecanoid pathway involving the action of lipoxygenase on membrane lipids; and in several plant systems take part in signalling pathways leading to flavanoids, terpenoids and coumarins such as scopoletin, which may function as phytoalexins.

Comparison of MecCAT1 transcript expression, and overall relative catalase enzyme activity as measured by tissue printing, indicated higher levels of catalase were associated with reduced susceptibility to PPD. A similar role for catalase has been demonstrated in senescence of muskmelon fruits (Laccan and Baccou 1998). In a comparative study on a two varieties showing contrasting storage life, higher sustained levels of catalase activity were found in the long storage life variety. Similarly, catalase activity levels have been shown to decline in tissues undergoing the HR (Adam *et al.* 1995, Milosovic and Slusarenko 1996).

CHAPTER FOUR:
STUDIES ON THE ROLE OF PEROXIDASES DURING POST-HARVEST PHYSIOLOGICAL DETERIORATION

4.1 Introduction and Literature Review

4.1.1 Types and classification of plant peroxidases

Peroxidases are a diverse family of enzymes which catalyse one electron oxidations using H_2O_2 as the electron acceptor and a wide variety of organic or inorganic substrates ranging from glutathione, ascorbate, cytochrome c, and NADH; to macromolecules such as proteins and polysaccharides as the electron donor (Lagrimi *et al* 1997, Asada 1992). The overall peroxidatic reaction may be summarised as below:

$$H_2O_2 + 2HA \rightarrow 2H_2O + 2A$$

where HA represents the electron donor. Broadly speaking peroxidases may be subdivided into two groups:

i) those peroxidases whose primary function lies in the scavenging of H_2O_2 or organic hydroperoxides,

ii) those where the oxidation products of the electron donors play a physiological role.

The former participate in the scavenging of H_2O_2 or organic hydroperoxides, and are referred to in the literature as heme, flavin or seleno peroxidases on the basis of prosthetic group; or ascorbate (EC 1.11.1.11), glutathione (EC 1.11.1.9), cytochrome c (1.11.1.5) or NADH (EC 1.11.1.1) peroxidases on the basis of electron donor. In mammalian, fungal and prokaryotic systems the major hydrogen peroxide scavenging peroxidases are the selenoenzyme glutathione peroxidase, the heme containing cytochrome c peroxidase and the flavoenzyme NAD(P)H peroxidase respectively. In plants, the bulk of H_2O_2 scavenging activity is believed to be effected by ascorbate peroxidases (Asada 1992).

The second group comprises the classical secretory or guiacol peroxidases (donor:H_2O_2 oxidoreductase, EC. 1.11.1.7) so called since guiacol has been widely used as a colorimetric electron donor in experimental assays, giving a dark brown oxidation product. The guiacol peroxidases are monomeric, heme containing enzymes. They are often further subdivided in the literature into cationic (pI 8.1-11), moderately anionic (pI 4.5-6.5) and anionic (pI 3.5-4) peroxidases (Abrahams *et al.* 1996).

Most plants have at least 10-20 peroxidase isoforms. Many of these are encoded by divergent genes and may differ by more than 50% in their amino acid sequences (Welinder 1992a). Other isoforms may originate from the same gene product and differ

in post-translational processing, such as degree of N-glycosylation (Lagrimini 1997). The full number of peroxidase encoding genes in many plant systems is still unclear and may have been underestimated – for example, initial analysis of data from the *Arabidopsis* sequencing project indicated more than 50 discrete genes (Ostergaard 1998).

4.1.2 Properties and reactions catalysed by plant peroxidases

The reactions catalysed and some properties of peroxidases which have been described in plants are briefly summarised in table 4.1.1. Whilst peroxidases generally serve to break down hydrogen peroxide, its formation may be catalysed by cell wall bound peroxidase at the expense of NADH (Mader *et al.* 1980, Thompson *et al.* 1987, Campa 1991). The reaction is strongly stimulated by phenolic compounds and Mn^{2+}, and is believed to provide the hydrogen peroxide required as a substrate for peroxidase catalysed lignification reactions during cell wall biosynthesis.

Glutathione peroxidases have only recently been reported in plants (Criqui *et al.* 1992). The glutathione peroxidases show little sequence similarity to the guiacol and ascorbate peroxidases and show low levels of activity. In mammals – which lack ascorbate peroxidase - the glutathione peroxidases are thought to be primarily responsible for the bulk scavenging of hydrogen peroxide, however, in plants, this role is assigned to ascorbate peroxidases or catalase, and it is believed the enzyme may serve an alternate role such as hydroperoxide scavenging (Eshdat *et al.* 1997).

The ascorbate peroxidases, whilst related to the guiacol peroxidases show higher sequence similarity to fungal cytochrome c peroxidase and exhibit high substrate

Recommended name	Reaction catalysed	Prosthetic group	Glycosylation	Organism	Location in plants	EC number	Systematic name
Peroxidase	donor + H_2O_2 ⇔ oxidised donor + $2H_2O$	heme	yes	plants	vacuole, cell wall tonoplast	1.1.11.7	donor:H_2O_2 oxidoreductase
L-ascorbate peroxidase	L-ascorbate + H_2O_2 ⇔ dehydro - ascorbate + $2H_2O$	heme	no	plants algae cyano-bacteria	chloroplasts, microbodies, cytosol	1.1.11.11	L-ascorbate: H_2O_2 oxidoreductase
Glutathione peroxidase	2 glutathione + H_2O_2 ⇔ oxidised glutathione + $2H_2O$	selenium		animals plants	unknown - possibly chloroplast stroma.	1.1.11.9	glutathione: H_2O_2 oxidoreductase

Table 4.1.1 Properties of plant peroxidases.

specificity towards ascorbate. In contrast the plant secretory peroxidases show little substrate specificity (Welinder 1992a).

4.1.3 Functions of plant peroxidases

The guiacol peroxidases have been implicated in a wide variety of physiological processes including cell wall biosynthesis and wound healing (via crosslinking of cell wall components such as extensins, involvement in lignification/lignin crosslinking and participation in suberin synthesis); defence against pathogens, senescence, breakdown of IAA (indole acetic acid) and ethylene synthesis. (Roberts *et al.* 1988, Asada 1992, Campa *et al.* 1991, Bradley *et al.* 1992, Carpin *et al.* 1999, Quiroga *et al.* 2000). However, the precise role of individual isoforms is often unclear due to the lack of information on their localisation and knowledge of their substrate specificity. Individual isozymes display varying degrees of reactivity to different substrates and may show considerable overlap of activities, although some electron donors such as lignin and extensin may be oxidised by specific isoforms (Brownleader 1995, Roberts 1988, Carpin *et al.* 1999). Some authors have proposed that anionic/acidic peroxidases are associated with lignification, whilst cationic/basic peroxidases are most effective in IAA catabolism (Brownleader 1995). In addition, cationic guiacol peroxidases may act *in vivo* as ascorbate peroxidases, scavenging hydrogen peroxide formed in the intracellular space (Campa 1991).

4.1.4 Location and targeting of plant peroxidases

The guiacol peroxidases (EC 1.11.1.7) are secretory proteins. They are synthesised on the rough ER (endoplasmic reticulum) on bound ribosomes and enter the secretory pathway via the Golgi apparatus and Golgi derived vesicles. Histological and cytological studies at both the light and electron microscopy level, utilizing substrates such as DAB (diaminobenzidine), TMB (tetramethylbenzidine) and PPD-PC (paraphenyldiamine pyrocatechol), have indicated that the main compartments of localization and function are the cell wall and the vacuole - both of which are extra-cytoplasmic spaces of the plant cell. Activity has also been detected in elements of the secretory pathway (Campa 1991, Catesson 1992, Mader 1992, Crevecoeur *et al.* 1997). Most known peroxidases contain a signal peptide that directs the nascent protein into the ER. The signal sequence comprises a central region of hydrophobic amino acid residues, preceded or flanked by regions containing polar amino acid residues. Some contain in addition a C-terminal extension thought to direct the protein to the vacuole (Carpin

1999, Lewin 1996, Welinder 1992b). Several studies have suggested that acidic (anionic) peroxidases are predominantly localised to the cell wall, whilst basic (cationic) peroxidases are vacuolar or bound to the tonoplast (Campa 1991, Mader 1992, Brownleader 1995, Welinder 1992b).

4.1.5 Previous studies on peroxidases during post-harvest physiological deterioration of cassava storage roots

A possible role for peroxidases during post-harvest physiological deterioration of cassava storage roots was first postulated by Czyhrinciw and Jaffe (1951), who proposed catalases, peroxidases and dehydrogenases as candidate enzymes. Work by Averre (1967) confirmed that the response was enzymatic in nature, however, detailed studies on peroxidases during cassava PPD were not carried out until the 1980s. In the first detailed study on cassava peroxidases, Marriot and colleagues reported an increase in total peroxidase activity following initiation of deterioration, with the appearance of a new band and intensification of others on electrophoretic separation. In addition, vacuum infiltration of horseradish peroxidase led to increased symptoms of PPD (Marriot *et al.* 1980). In further studies (Plumbley *et al.* 1981) peroxidase activity in soluble, covalently bound and ionically bound fractions of protein extracts from deteriorated and non-deteriorated root tissue were compared using enzyme assays and polyacrylamide gel electrophoresis (PAGE). Peroxidase activity as determined by the enzyme assay was 1.5 – 2 times higher in deteriorated tissue compared to non-deteriorated tissue in all 3 fractions. On polyacrylamide gel electrophoresis, deteriorated tissue showed 9, 2 and 3 activity bands in the soluble, ionically bound and covalently bound fractions respectively. Non-deteriorated tissue showed 8 soluble, 1 covalently bound and no ionically bound activity bands. Such bands may correspond to separate individual isoforms, however artefacts resulting from extraction procedures such as acid shocking may give rise to several activity bands from a single peroxidase. Changes were observed in the electrophoretic pattern of soluble peroxidases extracted over a period of 22 hours after mechanical damage, with a novel isoforms (R_f 0.3) first detected at 12 hours after injury and increasing markedly during the timecourse. Application of exogenous ethylene led to increased staining of this band. In the same study, ethylene production from injured cassava roots was shown to increase steadily after a lag of 6 hours, and the authors speculate that this increase in ethylene may affect tissue discoloration by altering peroxidase regulation.

Later studies comparing soluble peroxidase isoforms in deteriorated and non deteriorated root samples (Plumbley and Hughes 1982, Padmaja and Balogopal 1985) gave essentially similar results with the R_f = 0.3 novel isoform again detected in deteriorated root in both studies.

These results were confirmed and elaborated in subsequent studies (Uritani *et al.* 1983, Tanaka *et al.* 1983, Rickard and Gahan 1983). In a study on secondary metabolites and enzymes relating to their formation or turnover during PPD of cassava storage roots, Uritani and colleagues (Uritani *et al.* 1983) confirmed the increase in peroxidase activity throughout the root parenchyma during the response. Total peroxidase as measured by enzyme assays was low in cassava roots initially after harvest and continued to increase in all parts of the root parenchyma over a timecourse of 3 days. The peroxidase activity paralleled the degree of PPD observed, and was increased in response to mechanical injury. With regard to secondary metabolites, the authors report the identification of 3 bluish fluorescent coumarin components (scopoletin, scopolin and esculin) and 2 phenolic components (+ catechin and + gallocatechin) that were produced during the response. Scopolin, scopoletin and esculin were hardly present in fresh roots and accumulated in response to harvesting and cut injury over a timecourse of 60 hours. Scopoletin was produced first, reaching a peak at 20 hours before declining, and appeared to be converted to scopolin which continued to accumulate until 50 hours incubation before declining. Both the coumarin and the phenolic components were present predominately in and around areas of vascular discoloration and tissue browning. The authors proposed that the symptoms of PPD result from the oxidation of phenolic components such as (+) catechin by peroxidase and or polyphenol oxidase, resulting in the formation of insoluble coloured quinines and their reaction products which are known to be responsible for the black and brown pigments observed in many plants following injury or harvest (Uritani *et al.* 1983, Tanaka *et al.* 1983, Kahn 1977, Laukkanen *et al.* 1999).

In the first detailed cytological examinations Rickard (1982, Rickard *et al.* 1979) reported the occurrence of coloured amber brown/green brown xylem occlusions macroscopically observed as vascular streaking. In deteriorating tissue the site of production was in adjacent xylem parenchyma cells, entering the xylem vessels via pit areas. The exact identity of the pigmented material was not determined, however they were found to contain lipids, carbohydrates and phenolic material. Increases in peroxidases and polyphenol oxidase activities were again noted. (Rickard *et al.* 1983, Rickard 1985).

Studies by several authors (Wheatley 1982, Data *et al.* 1984, Tanaka *et al.* 1983, Tanaka *et al.* 1984) had suggested that roots from pruned plants were less susceptible to deterioration. Studies by Tanaka *et al.* (1984) and subsequent research has indicated that both peroxidase activity and coumarin components were markedly lower in roots from pruned plants over a 2 day time course (Tanaka *et al.* 1984, Kato *et al.* 1991). Campos and Carvalho (1990) compared levels of peroxidase activity and of phenolic components in roots of 3 cultivars showing differing susceptibility to PPD. Over a 7 day time course peroxidase levels as measured by enzyme assays were significantly higher in the susceptible cultivar Sonora than in the less susceptible cultivar Gauxupe. Phenolic compounds were likewise higher in the less susceptible cultivar Sonora.

4.1.6 Chapter summary

The isolation and characterisation of cDNAs encoding peroxidase isoforms expressed during PPD is described. Peroxidase enzyme activity and localisation at the tissue and cellular level over the post-harvest period was examined using tissue printing and light microscopy techniques. Isoforms of peroxidase during the post harvest period were examined by polyacrylamide gel electrophoresis. The isolated cDNA clones were used to study the expression of these peroxidase isoforms in different tissues and in response to treatments such as pre-harvest pruning, ethylene and the wound signal associated plant hormone jasmonic acid. Expression during the post harvest period was examined in a range of cultivars showing differing susceptibility to PPD.

4.2 Isolation and characterization of peroxidase cDNAs expressed during PPD of cassava storage roots.

Initial attempts to isolate and characterize cassava peroxidase cDNAs expressed during PPD were based on a heterologous screening approach. The post-harvest root cDNA library constructed in λgt10 (Beeching *et al.* 1997) was screened as described in section 2.7.6. The available peroxidase cDNAs (section 2.3) were used as probes either singly or as a mixture of similar probes. Probe DNAs were grouped together on the basis of an alignment of the available probe DNAs, using the PILEUP programme within the gcg (genetics computer group, Wisconsin) package (Devereux *et al.* 1984) and fell into 3 discrete groups (data not shown). Various hybridisation temperatures and final wash stringencies were employed, ranging from 60°C 1X SSC (highest stringency used) to 52 °C 3X SSC (lowest stringency used). However all experiments were unsuccessful giving either 0 positives or several false positives at lower stringencies. This lack of success

may have been due to the low degree of sequence similarity often noted between plant peroxidases (Welinder 1992a,b, Simon *et al.* 1996).

As an alternate strategy 2 cassava specific peroxidase probes, designated RPX and FPX, were generated by PCR. The oligonucleotide primer 5' - ACG AAG CAG TCG TGG AA -3' (PX-oligo) is homologous to a conserved active site region found in all secretory plant peroxidases and had been previously used as an oligonucleotide probe to isolate buffel grass peroxidases from a cDNA library (Ross *et al.* 1995). This primer was used to carry out 2 PCR reactions using a 5µl aliquot of the cassava root cDNA library as template DNA. For PCR reaction 1 (FPX), the PX-oligo was used in combination with the λgt10 forward primer (New England Biolabs); for PCR reaction 2 (RPX) it was used in combination with the λgt10 reverse primer (New England Biolabs). The λgt10 primers have binding sites approximately 100bp either side of the unique *Eco*RI insertion site used for cDNA insert ligation during cDNA library construction. Since the library is not directional this would allow amplification of peroxidase cDNA inserts which may be ligated into the λgt10 vector in either orientation. The strategy was intended to fulfil 2 aims – firstly to confirm the presence of cassava peroxidase cDNAs containing the PX-oligo motif in the cDNA library prior to screening; and secondly to generate specific cassava peroxidase probes which could be used to screen the cDNA library. Results of the PCR amplification reactions are shown in figure 4.2.1. Both reactions generated a single strong band of approximately 300bp. The PCR products were excised from the gel, purified as described in section 2.7.3. and were then sequenced on an ABI 337 automated fluorescent sequencer using the PX-oligo as primer. Sequence data was submitted to a blastx search using the NCBI database (www.ncbi.nlm.nih.gov). Both PCR products showed strong homology to plant peroxidase sequences. When the cassava FPX and RPX PCR sequences were compared using the "blast 2 sequences" programme on the NCBI database the degree of similarity in the overlapping region was high (96%), indicating that the cognate mRNAs probably encoded similar or identical cassava peroxidase transcripts which had been cloned into λgt10 in different orientations.

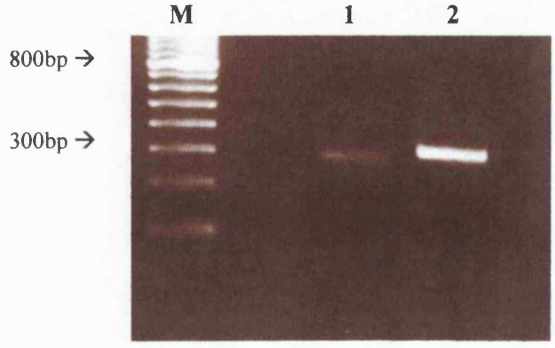


Figure 4.2.1 Cassava peroxidase PCR products. FPX and RPX electrophoresed on a 2% TAE gel. M = marker DNA (100bp ladder), 1 = FPX PCR product, 2 = RPX PCR product.

For screening of the cDNA library, the FPX and RPX PCR products were initially digested with *Eco*RI in order to remove λgt10 sequences present in the probe.
Restriction digest reactions were electrophoresed on a 2% agarose gel and the required bands were purified from the gel.

Hybridisation screening of the cDNA library was carried out using the FPX probe at high stringency (final wash conditions 60°C in 0.5X SSC). After 2 rounds of hybridisation a single positive clone designated MecPX1 (*Manihot esculenta* cDNA encoding peroxidase 1) was isolated. Lambda DNA from the positive plaque was amplified and purified as described in section 2.7.8 and the cDNA insert was excised by restriction digestion with *Eco*RI. The insert was purified from an agarose gel and subcloned into the *Eco*RI site of the plasmid vector pUC18. Initial sequencing was carried out using pUC/M13 forward and reverse primers, for subsequent sequencing reactions primers (PX1F2 and PX1R2) were designed using the Primer Designer for Windows programme (Scientific and Educational Software). Overlapping sequence data for both strands was compiled using the gelstart set of programmes within the gcg package (Devereux *et al.* 1984) as shown in figure 4.2.2.

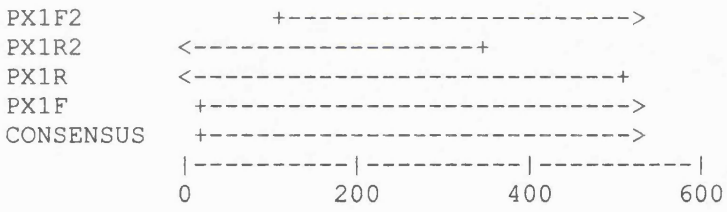


Figure 4.2.2 Diagrammatic representation of the sequencing strategy used for sequencing of MecPX1

The sequence and deduced translation of MecPX1 are shown in figure 4.2.3. Subsequent analysis of the nucleotide sequence and deduced translation was carried out either "by eye" or using the following programmes- ORF Finder (www.ncbi.nlm.nih.gov/gorf/orfig/cgi); P-SORT Prediction of Protein Localisation Sites v6.4 (psort.nibb.ac.jp); Predict Protein (www.embl.heidelberg), gcg (genetics computer group, Wisconsin, Devereux *et al.*, 1984) SignalP (genome.cbs.dtu.dk, Nieilsen *et al.* 1997) and the GENEDOC package (Nicholas and Nicholas, 1997). The isolated clone was a partial peroxidase cDNA of 556bp in size and encoded a predicted protein of size 184 amino acids – approximately half of the size expected for a full length plant peroxidase. Five of the eight conserved Cys residues found in other peroxidases, known

to be involved in disulphide bridge formation in the mature protein , were present in the sequence. The predicted polypeptide contained a short (21 amino acid) signal sequence for direction of the nascent polypeptide into the endoplasmic reticulum with a predicted cleavage site between residues 21 and 22 (Ala - Gln) (Nieilsen *et al.* 1997). Virtually all proteins that pass through the secretory apparatus are glycosylated by the addition of an oligosaccharide moiety to the NH_2 group of asparagines (N-linked glycosylation) or the OH group of serine or threonine (O-linked glycosylation). MecPX1 contains 3 putative consensus glycosylation sites (consensus Asn-X-Ser/Thr) as shown in figure 4.2.3.

```
1   aatcttttctctcctttgatctcttgtattttggttgcttcttgctttgc
       I  F  S  L  L  L  I  S  C  I  L  V  A  S  C  F
52  tgccattgaagcacaaaagagacctcctatagtgaatggtctctcatggaa
    A  A  I  E  A  Q  K  R  P  P  I  V  N  G  L  S  W
103 tttctacaaaactagttgccctaaagttgaatccatcatcaaaaatgaact
    N  F  Y  K  T  S  C  P  K  V  E  S  I  I  K  N  E
154 caagaaagttttcaagaaggatattggtcaagctgctggcgttcttcgtat
    L  K  K  V  F  K  K  D  I  G  Q  A  A  G  V  L  R
205 acagttccacgactgcttcgttcagggatgcgatggctcggtgctgcttga
    I  Q  F  H  D  C  F  V  Q  G  C  D  G  S  V  L  L
256 tggatcagcaggcggaccaagcgagaaatctgaacttccgaacttgacttt
    D  G  S  A  G  G  P  S  E  K  S  E  L  P  N  L  T
307 gagaaaagaggcatttaaaatcatcaacgacctccgcgatgctgtccacaa
    L  R  K  E  A  F  K  I  I  N  D  L  R  D  A  V  H
358 gcagtgtggccgtgtcgtttcttgctctgacatcgtcgccatcgccgcccg
    K  Q  C  G  R  V  V  S  C  S  D  I  V  A  I  A  A
409 tgactccgttgtcttgaccggcggtcctgactacgatgtccccttaggaag
    R  D  S  V  V  L  T  G  G  P  D  Y  D  V  P  L  G
460 gcgagacggtgtcgtatttgctcaagtaaaccaaacttttatcgacctggt
    R  R  D  G  V  V  F  A  Q  V  N  Q  T  F  I  D  L
511 aggacctgatgctaacactactaccatcctcaccaagcttgcccc 556
    V  G  P  D  A  N  T  T  T  I  L  T  K  L  A
```

Figure 4.2.3 Sequence and deduced translation of MecPX1. The predicted signal sequence is shown in blue with yellow text, conserved Cys residues are shown in red, potential glycosylation sites are highlighted in pale blue. The predicted cleavage site for the signal peptide is shown in bold. Within the nucleotide sequence, the binding site for the primer, PX-oligo, is shown in bold.

Screening of the cDNA library at high stringency using RPX as the probe gave 3 duplicate positive plaques after 2 successive rounds of hybridisation. These were designated MecPX2, MecPX3, and MecPX4. The cDNA inserts from the positive plaques were amplified by PCR and sequenced using the λgt10 forward or reverse primer as previously described. The PCR sequence data was aligned with MecPX1 using the gcg pileup programme in order to determine the relatedness of the isolated peroxidase cDNAs. Part of the alignment is shown below (figure 4.2.4). These data

indicate the clones obtained with the RPX probe were largely identical to the MecPX1 clone previously isolated, although they differed at the extreme 5' end. The nucleotide differences observed in MecPX4 could reflect errors introduced during PCR, however they may indicate that MecPX4 is similar but not identical peroxidase to MecPX1.

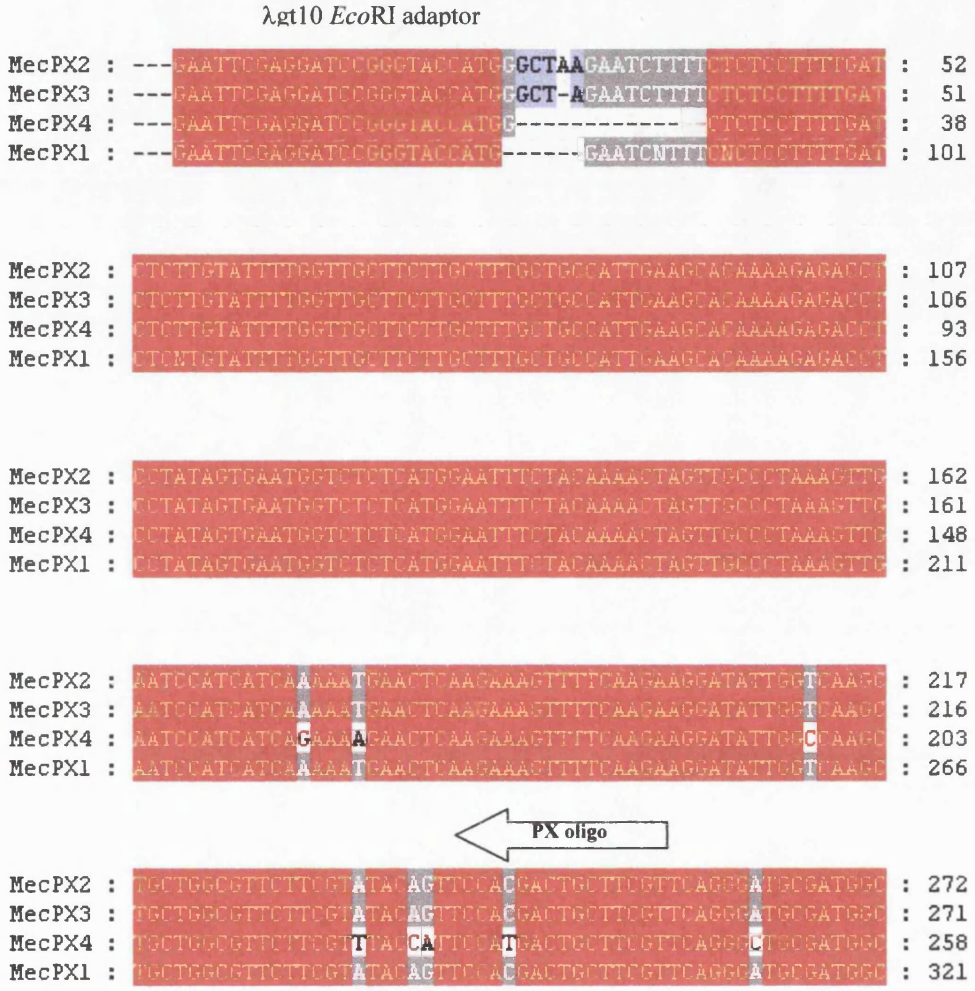


Figure 4.2.4 Partial nucleotide alignment of cassava peroxidase sequences. Colour blocking indicates the degree of sequence conservation. Red = 100% Identity. Grey = >70% identity. Blue = > 50% identity. The region corresponding to the binding site for PX oligo is indicated by an arrow.

Since clone MecPX2 was the largest of the three novel peroxidase clones obtained, it was selected for further study and was subcloned into the *Eco*RI site of pUC18 and sequenced as described for MecPX1. A diagrammatic representation of the sequencing strategy used is shown in figure 4.2.5. The nucleotide sequence and deduced translation of MecPX2 is shown in figure 4.2.6.

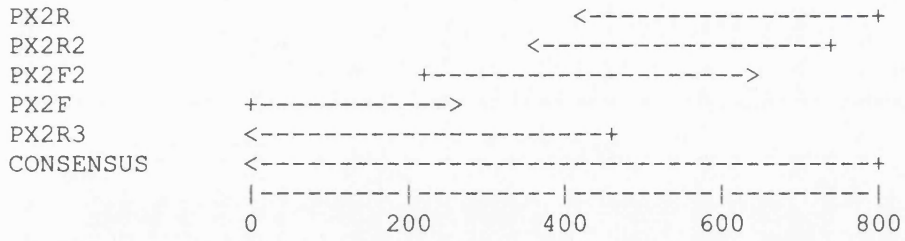


Figure 4.2.5 Diagrammatic representation of the sequencing strategy used for sequencing of MecPX2

Subsequent analysis of the nucleotide sequence and deduced translation was carried out as described for MecPX1 using ORF Finder (www.ncbi.nlm.nih.gov/gorf/orfig/cgi); P-SORT Prediction of Protein Localisation Sites v6.4 (psort.nibb.ac.jp); Predict Protein (www.embl.heidelberg), gcg (genetics computer group, Wisconsin, Devereux *et al.*, 1984) and SignalP (genome.cbs.dtu.dk, Nieilsen *et al.* 1997).The clone is 726bp in size and encodes a predicted protein of size 244 amino acids. It was identical to MecPX1 but contained an additional 170bp, with additional sequence data at both the 3' and 5' ends.

```
1     gctagaatcttttctctccttttgatctcttgtattttggttgcttcttgctttgctgcc
      A  R  I  F  S  L  L  L  I  S  C  I  L  V  A  S  C  F  A  A
61    attgaagcacaaaagagacctcctatagtgaatggtctctcatggaatttctacaaaact
      I  E  A  Q  K  R  P  P  I  V  N  G  L  S  W  N  F  Y  K  T
121   agttgccctaaagttgaatccatcatcaaaaatgaactcaagaaagttttcaagaaggat
      S  C  P  K  V  E  S  I  I  K  N  E  L  K  K  V  F  K  K  D
181   attggtcaagctgctggcgttcttcgtatacagttccacgactgcttcgttcagggatgc
      I  G  Q  A  A  G  V  L  R  I  Q  F  H  D  C  F  V  Q  G  C
241   gatggctcggtgctgcttgatggatcagcaggcggaccaagcgagaaatctgaacttccg
      D  G  S  V  L  L  D  G  S  A  G  G  P  S  E  K  S  E  L  P
301   aacttgactttgagaaaagaggcatttaaaatcatcaacgacctccgcgatgctgtccac
      N  L  T  L  R  K  E  A  F  K  I  I  N  D  L  R  D  A  V  H
361   aagcagtgtggccgtgtcgtttcttgctctgacatcgtcgccatcgccgcccgtgactcc
      K  Q  C  G  R  V  V  S  C  S  D  I  V  A  I  A  A  R  D  S
421   gttgtcttgaccggcggtcctgactacgatgtccccttaggaaggcgagacggtgtcgta
      V  V  L  T  G  G  P  D  Y  D  V  P  L  G  R  R  D  G  V  V
481   tttgctcaagtaaaccaaacttttatcgacctggtaggacctgatgctaacactactacc
      F  A  Q  V  N  Q  T  F  I  D  L  V  G  P  D  A  N  T  T  T
541   atcctcaccaagcttgcccggaaaaatcttgacgccaccgatgcggtggccctctcaggc
      I  L  T  K  L  A  R  K  N  L  D  A  T  D  A  V  A  L  S  G
601   gcccacaccatcggcattggccactgcacatctttcactgaccgtctttatccaacccaa
      A  H  T  I  G  I  G  H  C  T  S  F  T  D  R  L  Y  P  T  Q
661   gatcctaccttggacaaaacatttgccaacaatctcaagcgaacttgtccaaaagaagac
      D  P  T  L  D  K  T  F  A  N  N  L  K  R  T  C  P  K  E  D
721   accacc
      T  T
```

Figure 4.2.6 Sequence and deduced translation of MecPX2. The predicted signal sequence is shown in blue with yellow text, conserved Cys residues are shown in red, potential glycosylation sites are highlighted in pale blue. The predicted cleavage site for the signal peptide is shown in bold. The region corresponding to the binding site for PX oligo within the nucleotide sequence is shown in bold.

MecPX2 again represents a partial peroxidase cDNA although additional sequence data relative to MecPX1 are present. The 5' end of the clone contains an additional 6 nucleotides, and slightly extends the predicted N-terminal signal sequence of the peroxidase for co-translational direction of the nascent polypeptide into the lumen of the endoplasmic reticulum (ER). Such signal sequences comprise 15 – 30 amino acids with a central region of predominantly hydrophobic residues (Ala, Val, Leu, Ile, Pro, Trp, Phe, Met); preceded or flanked by polar residues (Gly, Ser, Thr, Tyr, Cys, Asn, Gln) but show little other sequence conservation. The leader sequence of the peptide serves to promote attachment of the peptide and the ribosome translating it to the ER membrane, during translation the peptide chain is directed through the membrane to the ER lumen, where the signal sequence is cleaved by a protease associated with the membrane. The predicted cleavage site of the pre-protein encoded by MecPX2 was again located between residues 21 and 22 (Ala - Gln) using the SignalP V2 software (http://genome.cbs.dtu.dk/htbin/nph-webface) (Nieilsen *et al.* 1997). The predicted leader sequence of MecPX2 is 24 residues in length, however an initiating Met is not present in the sequence suggesting the clone is truncated at both the 5' and 3' ends. MecPX2 contains seven of the eight conserved Cys residues found in other peroxidases, and one additional predicted N-glycosylation site relative to MecPX1. The predicted pI of the pre-peptide when submitted to the ProtParam tool (http://expasy.cbr.nrc.ca/cgi-bin/protparam) was 8.9. Although the predicted pI would be altered by the presence of 3' residues which are missing in the partial clone, these data suggest that MecPX1 and MecPX2 may encode a basic/cationic cassava peroxidase.

4.3 Comparative sequence analysis

A vast number of peroxidase sequences including numerous short ESTs have been deposited with the NCBI database, indeed a search using the terms "viridiplantae" + "peroxidase" generated over 1000 entries. The amino acid sequences for 34 plant peroxidases were chosen essentially at random, although a single previously isolated partial cassava peroxidase sequence (Pereira *et al.*, unpublished), and 2 sequences from azuki bean (*Vincula ungulata*) and flax (*Linum usitatissimum*) which had shown highest similarity to MecPX2 in a blastx search were specifically chosen. Sequences were aligned using the CLUSTALW programme (Thompson *et al.* 1994) within gcg. Alignments in PHYLIP interleaved format (Felsenstein, 1994) were used to construct an unrooted tree by the neighbour joining method using the Tajima and Nei algorithm

(Tajima and Nei, 1984) with the TreeCon 1.2 package (Van de Peer and De Wachter 1994). The dendrogram is shown in figure 4.3.1. The sequences grouped into a large number of small clades and the overall tree topology showed large distances reflecting the low degree of sequence conservation among plant peroxidases. As would be expected, the ascorbate peroxidases grouped separately supported by high bootstrap values. MecPX1 and MecPX2 grouped as expected with a wound and ethylene induced, vacuolar cationic peroxidase from azuki bean (*Vingula angularis*) (Ishige *et al.* 1993) and the flax (*Linum usitatissimum*) sequence for which no other details were available.

The alignment used in tree construction is shown in figure 4.3.2. The predicted amino acid sequences of MecPX1 and MecPX2 are shown in a ClustalW alignment with 34 plant peroxidase sequences. Overall similarity between sequences in the alignment used for tree construction was 35 – 65%. Similarity of the deduced MecPX2 pre-protein to the previously isolated partial cassava peroxidase of Pereira *et al.* was 41% over the overlapping region. Similarity to the azuki bean cationic peroxidase was 61%. Interestingly, all 3 sequences to which MecPX1 and MecPX2 show highest similarity to – azuki bean (*V.angularis*) VIRPRX, flax (*L.usitatissimum*) flxper3, and thale cress (*Arabidopsis thaliana*) prxr6 - contain C-terminal extensions relative to other peroxidases. These C terminal extensions are similar to the C terminal extensions found in vacuolar proteins and contain predominantly hydrophobic residues (Abrahams *et al.* 1996, Carpin 1999). Taken together with the predicted cationic pI, this suggests that the peroxidase encoded by MecPX1 and MecPX2 may be localised to the vacuole in cassava storage root cells. It should be noted however that similar C-terminal extensions were found in 2 anionic peroxidases – parsley (*Petroselium crispum*) and pumpkin (*Cucurbita pepo*), whilst they were absent in a number of cationic peroxidases including those of chickpea (*Cicer arietum*) and tobacco (*Nicotiana tabacum*).

The general structure of plant peroxidases – comprising 3 conserved domains interspersed with non-conserved domains is evident from figure 4.3.2. The first domain (A) has the core consensus FHDCFV and comprises the catalytic and distal heme binding domain. The third domain (C) has the core consensus ALSAGAHT and forms the proximal heme binding domain. The central conserved domain (B) is of unknown function, but is thought to be involved in maintaining the 3 dimensional structure of the protein (Welinder 1985, Abrahams *et al.* 1996).

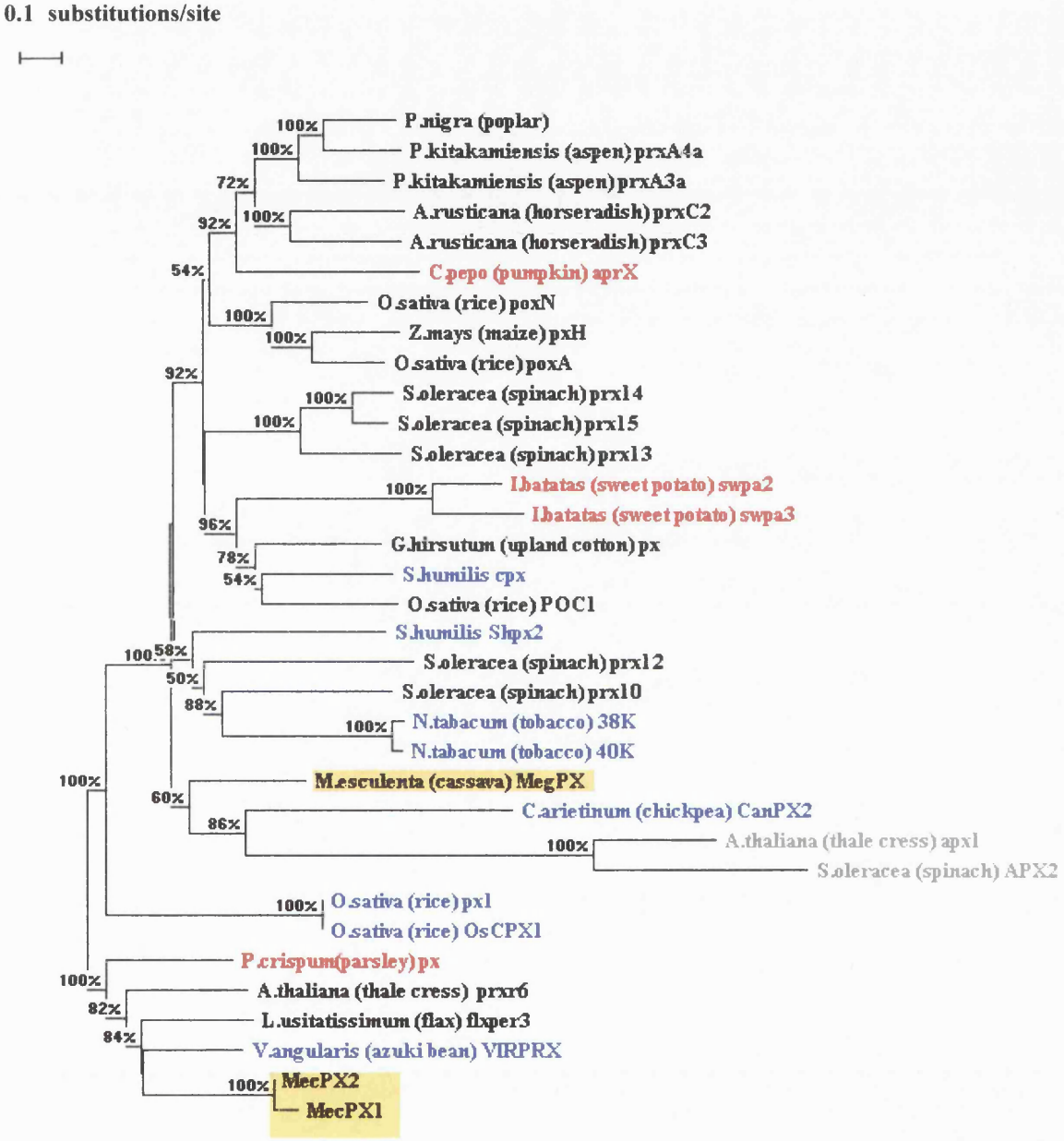
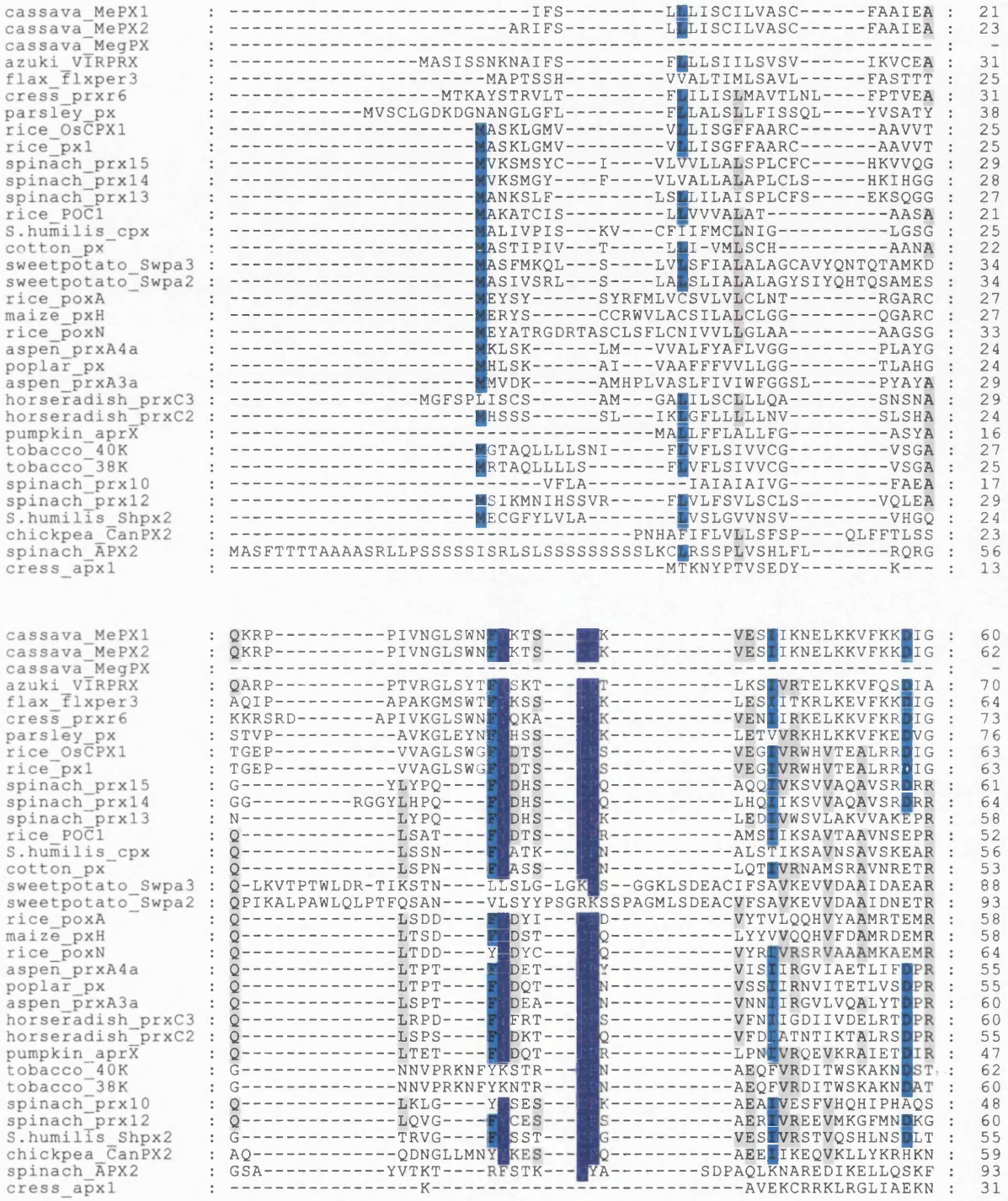
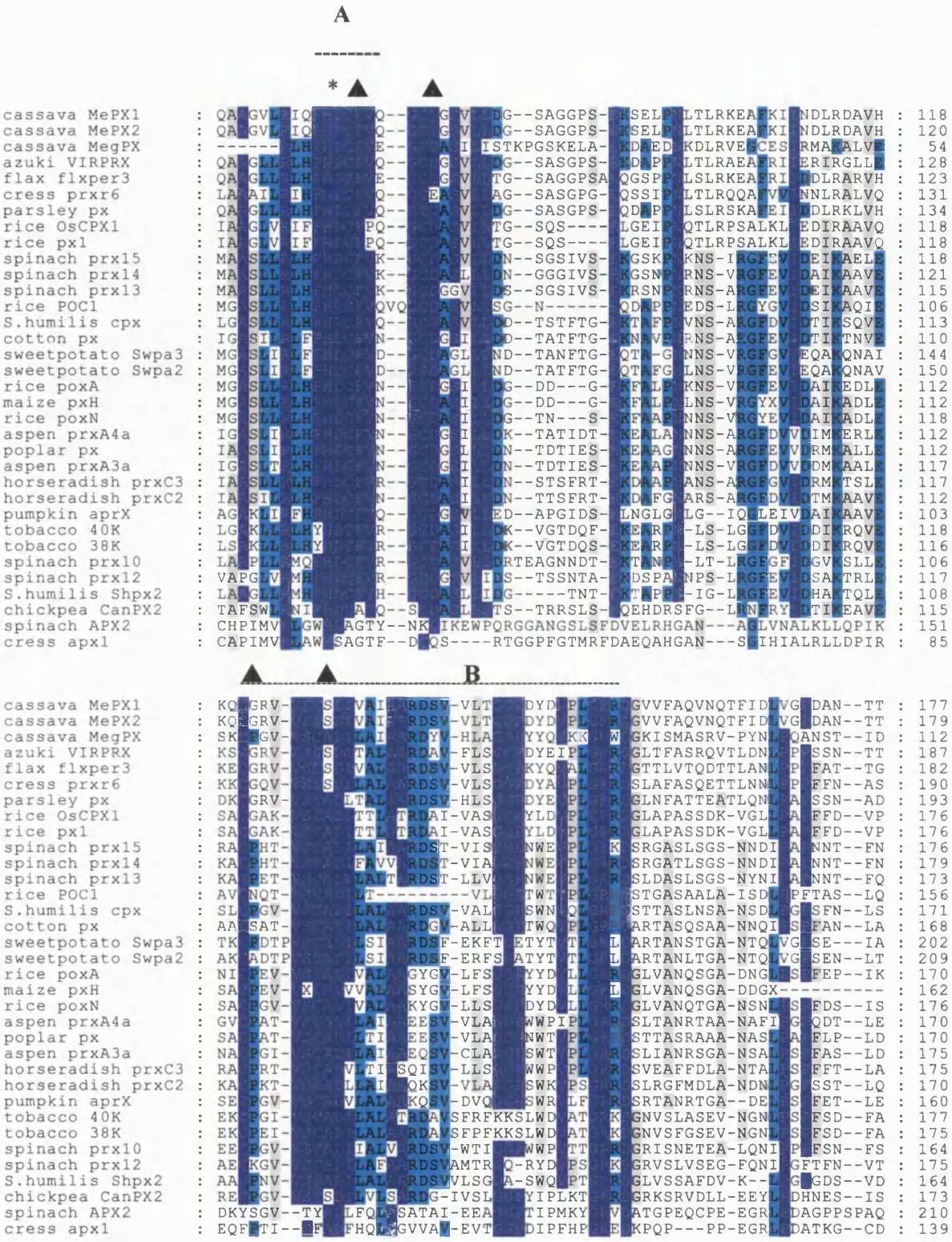
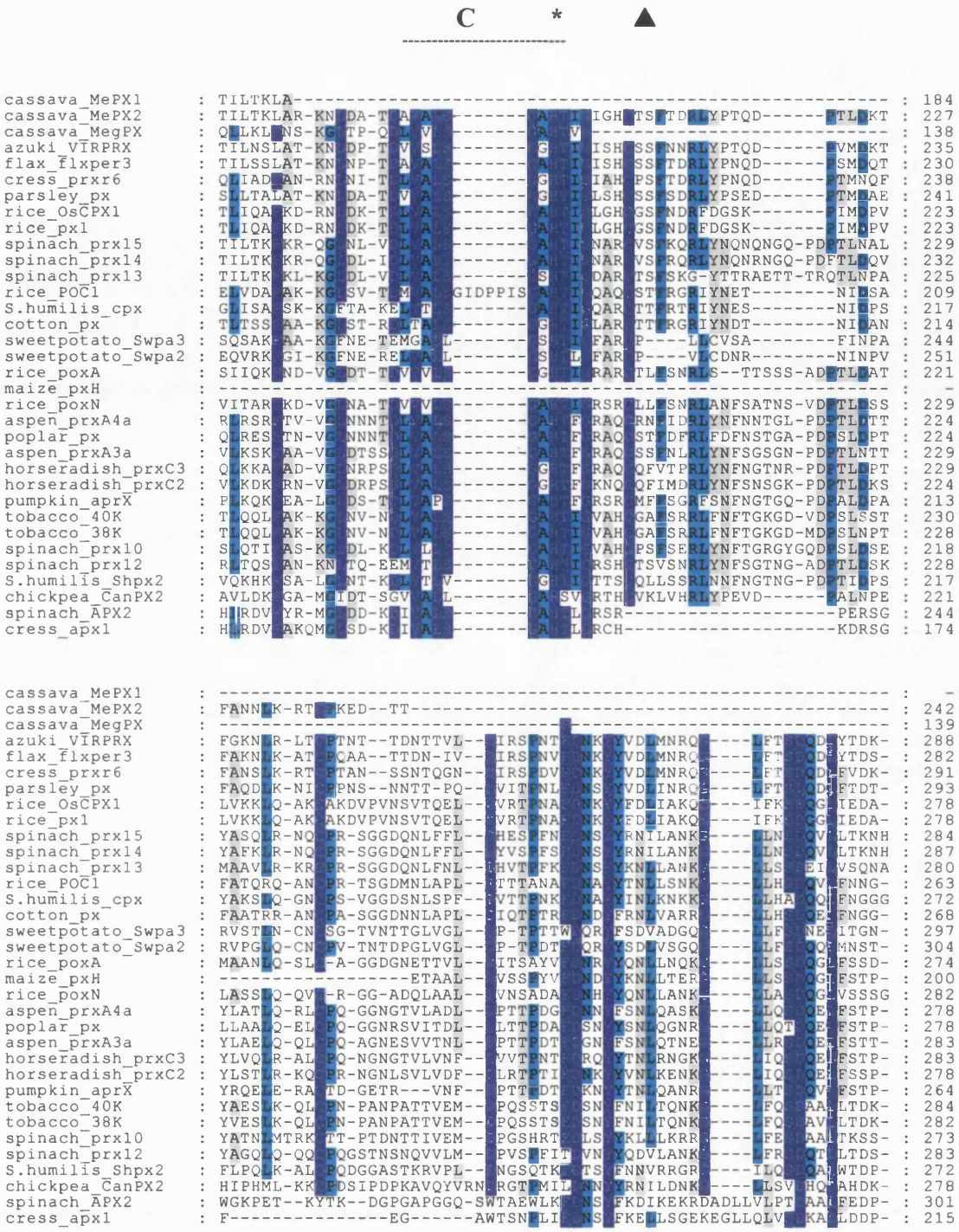


Figure 4.3.1 Unrooted tree constructed from 34 plant peroxidase amino acid sequences using the Tajima and Nei algorithm (TreeCon package). Branch lengths reflect the number of substitutions per 1000 residues. Bootstrap values greater than 50% for 100 replicate samples are shown at the nodes. Where known, basic/cationic sequences are shown in blue, acidic/anionic sequences are shown in red. Ascorbate peroxidase sequences are shown in grey. Cassava sequences are shown in yellow blocks.


cassava_MePX1 : ----------IFS---------LLLISCILVASC------FAAIEA : 21
cassava_MePX2 : ----------ARIFS---------LLLISCILVASC------FAAIEA : 23
cassava_MegPX : ---------- : -
azuki_VIRPRX : ----------MASISSNKNAIFS---------FLLLSIILSVSV------IKVCEA : 31
flax_flxper3 : ----------MAPTSSH---------VVALTIMLSAVL------FASTTT : 25
cress_prxr6 : ----------MTKAYSTRVLT---------FLILISLMAVTLNL----FPTVEA : 31
parsley_px : ----------MVSCLGDKDGNANGLGFL---------FLLALSLLFISSQL----YVSATY : 38
rice_OsCPX1 : ----------MASKLGMV---------VLLISGFFAARC-------AAVVT : 25
rice_px1 : ----------MASKLGMV---------VLLISGFFAARC-------AAVVT : 25
spinach_prx15 : ----------MVKSMSYC---I----VLVVLLALSPLCFC-----HKVVQG : 29
spinach_prx14 : ----------MVKSMGY----F----VLVALLALAPLCLS-----HKIHGG : 28
spinach_prx13 : ----------MANKSLF---------LSLLILAISPLCFS-----EKSQGG : 27
rice_POC1 : ----------MAKATCIS---------LLVVVALAT----------AASA : 21
S.humilis_cpx : ----------MALIVPIS---KV---CFIIFMCLNIG---------LGSG : 25
cotton_px : ----------MASTIPIV---T-----LLI-VMLSCH----------AANA : 22
sweetpotato_Swpa3 : ----------MASFMKQL---S----LVLSFIALALAGCAVYQNTQTAMKD : 34
sweetpotato_Swpa2 : ----------MASIVSRL---S----LALSLIALALAGYSIYQHTQSAMES : 34
rice_poxA : ----------MEYSY------SYRFMLVCSVLVLCLNT--------RGARC : 27
maize_pxH : ----------MERYS------CCRWVLACSILALCLGG--------QGARC : 27
rice_poxN : ----------MEYATRGDRTASCLSFLCNIVVLLGLAA--------AAGSG : 33
aspen_prxA4a : ----------MKLSK------LM---VVALFYAFLVGG--------PLAYG : 24
poplar_px : ----------MHLSK------AI---VAAFFFVVLLGG--------TLAHG : 24
aspen_prxA3a : ----------MMVDK------AMHPLVASLFIVIWFGGSL------PYAYA : 29
horseradish_prxC3 : ----------MGFSPLISCS------AM---GALILSCLLLQA--------SNSNA : 29
horseradish_prxC2 : ----------MHSSS------SL---IKLGFLLLLLNV--------SLSHA : 24
pumpkin_aprX : ----------MALLFFLALLFG---------ASYA : 16
tobacco_40K : ----------MGTAQLLLLSNI-----FLVFLSIVVCG---------VSGA : 27
tobacco_38K : ----------MRTAQLLLLS-------FLVFLSIVVCG---------VSGA : 25
spinach_prx10 : ----------VFLA---------IAIAIAIVG---------FAEA : 17
spinach_prx12 : ----------MSIKMNIHSSVR-----FLVLFSVLSCLS-------VQLEA : 29
S.humilis_Shpx2 : ----------MECGFYLVLA---------LVSLGVVNSV---------VHGQ : 24
chickpea_CanPX2 : ----------PNHAFIFLVLLSFSP----QLFFTLSS : 23
spinach_APX2 : MASFTTTTAAAASRLLPSSSSSISRLSLSSSSSSSSSLKCLRSSPLVSHLFL-------RQRG : 56
cress_apx1 : ----------MTKNYPTVSEDY--------K--- : 13
cassava_MePX1 : QKRP----------PIVNGLSWNFLKTS---LLK----------VESLIKNELKKVFKKLIG : 60
cassava_MePX2 : QKRP----------PIVNGLSWNFLKTS---LLK----------VESLIKNELKKVFKKLIG : 62
cassava_MegPX : ---------- : -
azuki_VIRPRX : QARP----------PTVRGLSYTFLSKT---LLT----------LKSLVRTELKKVFQSLIA : 70
flax_flxper3 : AQIP----------APAKGMSWTFLKSS---LLK----------LESLITKRLKEVFKKLIG : 64
cress_prxr6 : KKRSRD-------APIVKGLSWNFLQKA---LLK----------VENLIRKELKKVFKRLIG : 73
parsley_px : STVP----------AVKGLEYNFLHSS---LLK----------LETVVRKHLKKVFKELVG : 76
rice_OsCPX1 : TGEP----------VVAGLSWGFLDTS---LLS----------VEGLVRWHVTEALRRLIG : 63
rice_px1 : TGEP----------VVAGLSWGFLDTS---LLS----------VEGLVRWHVTEALRRLIG : 63
spinach_prx15 : G----------YLYPQ----FLDHS---LLQ----------AQQLVKSVVAQAVSRLRR : 61
spinach_prx14 : GG---------RGGYLHPQ----FLDHS---LLQ----------LHQLIKSVVAQAVSRLRR : 64
spinach_prx13 : N----------LYPQ----FLDHS---LLK----------LEDLVWSVLAKVVAKEPR : 58
rice_POC1 : Q----------LSAT----FLDTS---LLR----------AMSLIKSAVTAAVNSEPR : 52
S.humilis_cpx : Q----------LSSN----FLATK---LLN----------ALSTIKSAVNSAVSKEAR : 56
cotton_px : Q----------LSPN----FLASS---LLN----------LQTLVRNAMSRAVNRETR : 53
sweetpotato_Swpa3 : Q-LKVTPTWLDR-TIKSTN----LLSLG-LGKLS--GGKLSDEACIFSAVKEVVDAAIDAEAR : 88
sweetpotato_Swpa2 : QPIKALPAWLQLPTFQSAN----VLSYYPSGRKSSPAGMLSDEACVFSAVKEVVDAAIDNETR : 93
rice_poxA : Q----------LSDD----FLDYI---LLD----------VYTVLQQHVYAAMRTEMR : 58
maize_pxH : Q----------LTSD----FLDST---LLQ----------LYYVVQQHVFDAMRDEMR : 58
rice_poxN : Q----------LTDD----YLDYC---LLQ----------VYRLVRSRVAAAMKAEMR : 64
aspen_prxA4a : Q----------LTPT----FLDET---LLY----------VISLIRGVIAETLIFLPR : 55
poplar_px : Q----------LTPT----FLDQT---LLN----------VSSLIRNVITETLVSLPR : 55
aspen_prxA3a : Q----------LSPT----FLDEA---LLN----------VNNLIRGVLVQALYTLPR : 60
horseradish_prxC3 : Q----------LRPD----FLFRT---LLS----------VFNLIGDIIVDELRTLPR : 60
horseradish_prxC2 : Q----------LSPS----FLDKT---LLQ----------VFDLATNTIKTALRSLPR : 55
pumpkin_aprX : Q----------LTET----FLDQT---LLR----------LPNLVRQEVKRAIETLIR : 47
tobacco_40K : G----------NNVPRKNFYKSTR---LLN----------AEQFVRDITWSKAKNLST : 62
tobacco_38K : G----------NNVPRKNFYKNTR---LLN----------AEQFVRDITWSKAKNLAT : 60
spinach_prx10 : Q----------LKLG----YLSES---LLK----------AEALVESFVHQHIPHAQS : 48
spinach_prx12 : Q----------LQVG----FLCES---LLS----------AERLVREEVMKGFMNLKG : 60
S.humilis_Shpx2 : G----------TRVG----FLSST---LLG----------VESLVRSTVQSHLNSLLT : 55
chickpea_CanPX2 : AQ----------QDNGLLMNYLKES---LLQ----------AEELIKEQVKLLYKRHKN : 59
spinach_APX2 : GSA----------YVTKT----RFSTK---LYA--------SDPAQLKNAREDIKELLQSKF : 93
cress_apx1 : ----------K----------AVEKCRRKLRGLIAEKN : 31


A
B


C
*
▲

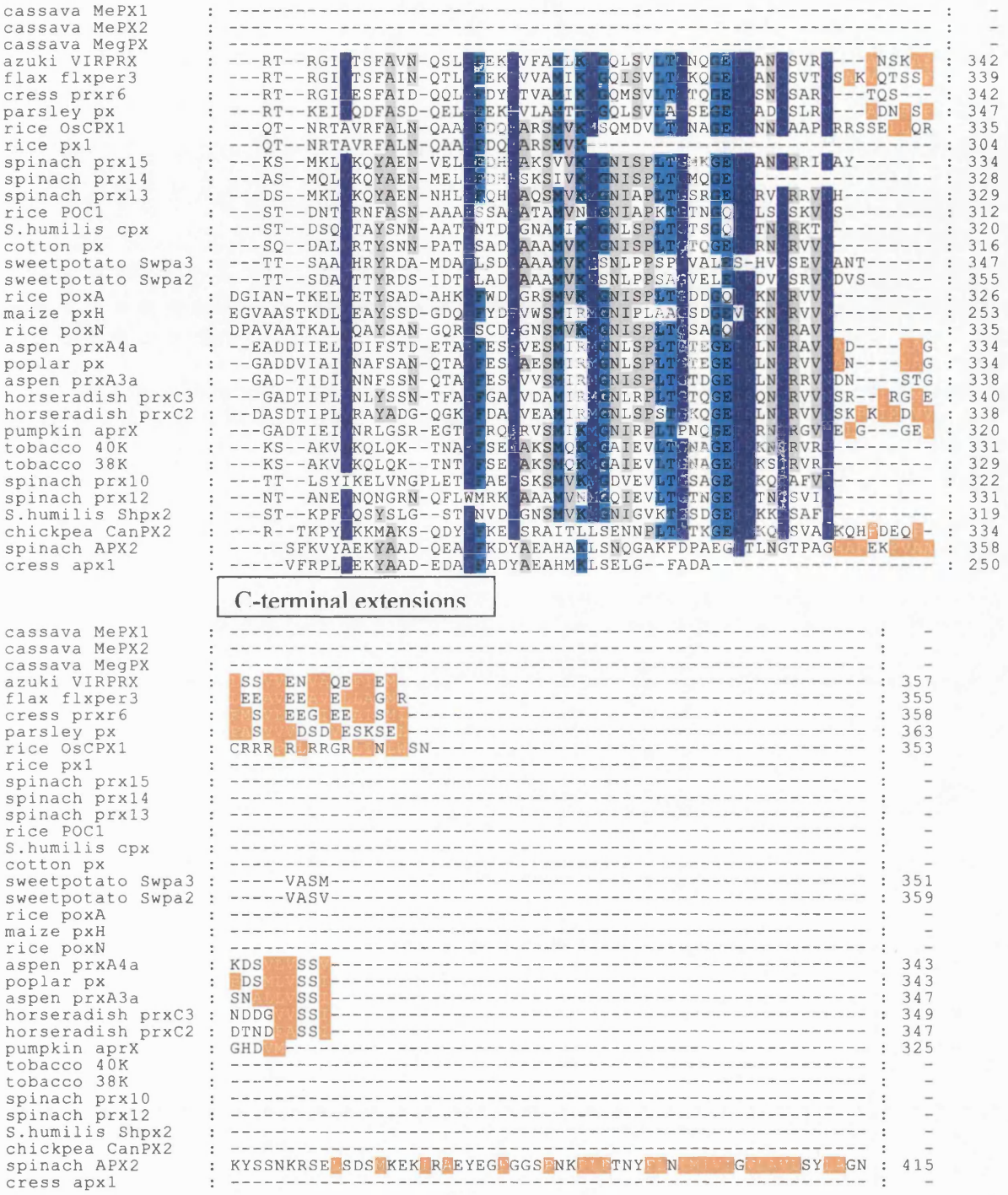


Figure 4.3.2 Amino acid alignment of 34 plant peroxidase sequences. Sequence conservation is indicated by colour blocking. Dark blue = > 80% identity, Light blue = > 60% identity, Grey = > 40% identity. The 2 conserved His residues involved in heme binding are indicated by an asterisk. The eight conserved Cys residues involved in disulphide bridge formation are indicated by triangles. The 3 conserved domains are indicated by lines above the sequence. A = catalytic and distal heme binding domain, B = conserved domain of unknown function, C = proximal heme binding domain. The C-terminal extensions thought to be involved in vacuolar targeting are indicated by a box. Hydrophobic residues are indicated in orange.

4.4 Peroxidase gene organisation in cassava

Southern blot hybridisations were carried out using the isolated cassava peroxidase cDNA clone MecPX1 as a probe. Hybridisations were carried out overnight at 55°C as described in section 2.7.9. For the low stringency wash membranes were washed twice for 20 minutes in 4X SSC, 0.1% SDS at 55°C. The high salt concentration was chosen as peroxidase sequences within the same organism may show considerable sequence divergence. For the high stringency wash, the membranes were washed twice for 20 minutes in 0.1X SSC, 0.1% SDS at 60°C. For the MecPX1 probe (of size 556bp) the minimum percentage homology required to allow stable probe-target hybridisation under low and high stringency conditions were 71% and 93% respectively, as calculated using the equation $Tm°C = 81.5°C + 16.6\log [Na^+] + 0.41(\%GC) - (600/1)$ as described in section 2.7.9 and assuming a GC content of 50%. Results are shown in figure 4.4.1.

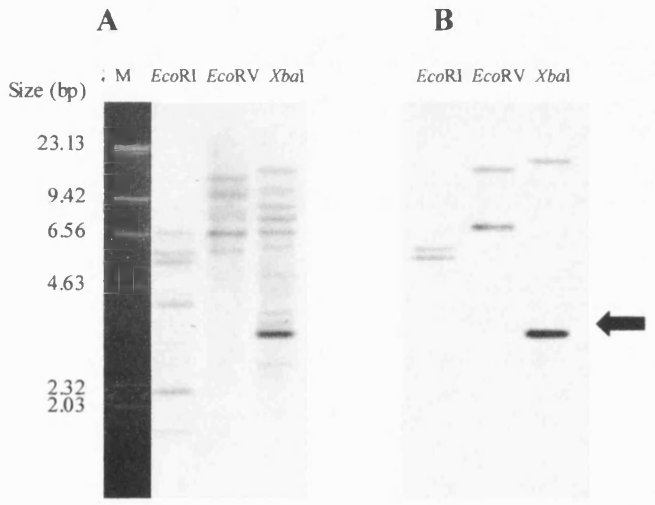


Figure 4.4.1 Southern blot hybridisation with MecPX1. 10μg of digested genomic DNA was run on a 0.8% TAE gel overnight and Southern blotted according to standard procedures. Hybridisation was carried out overnight at 55°C. Panel A = Low stringency wash (4X SSC, 0.1% SDS at 55°C for 2x 20 minutes). Panel B = High stringency wash (0.1X SSC, 0.1% SDS at 60°C for 2x 20 minutes).

For the low stringency wash a large number of hybridising bands of variable intensity were detected, 6-8 in the *Eco*RI lane, 5-7 in the *Eco*RV lane and at least 11 in the *Xba*I lane. The intensity of the band indicated by an arrow in figure 4.4.1 suggests it may comprise 2 hybridising bands of similar size. After washing at high stringency, 2 hybridising bands occur in each lane. A restriction map of the MecPX1 sequence used as a probe shows no internal restriction sites for the enzymes *Eco*RI, *Eco*RV and *Xba*I. However since the sequence is a cDNA no information is available on restriction sites

which may be present in intervening intron sequences. Therefore, these data indicate that MecPX1 forms part of a gene family in cassava, with at least 3 related peroxidase sequences showing > 71% homology to MecPX1. Note that the cassava peroxidase of Pereira *et al.* (unpublished) would not have been detected at this stringency, thus the actual number of peroxidase sequences present in the cassava genome may be significantly higher. For the high stringency wash the presence of 2 hybridising bands of similar intensity in the *Eco*RI and *Eco*RV lanes would be consistent with the presence of 2 copies of the MecPX1 sequence in the cassava genome, or the presence of a single gene containing both *Eco*RI and *Eco*RV restriction sites within the genomic intron sequences.

4.5 Detection of peroxidase isoforms by polyacrylamide gel electrophoresis

For the detection of cassava root peroxidase isoforms, total protein was extracted from air freighted roots of 2 cultivars- MCOL 22 and MPER 337 at 0, 1, 2, 3 and 4 days after injury. Protein extraction and purification was carried out as described in section 2.7.15. 30μg aliquots of each sample were then electrophoresed on an isoelectric focussing (IEF) polyacrylamide gel (Ampholine PAG plate pH 3.5 – 9.5 Amersham Pharmacia). After running the gel was stained for peroxidase activity. Two colorimetric substrates were used- 4-aminoantipyrine in combination with 3,5-dichloro-2-hydroxy benzene sulphonic acid (DHBS); and guiacol. The guiacol staining method (section 2.7.15.6) was found to give superior results with darker staining of activity bands and allowed visualisation of several bands that could not be detected by 4-aminoantipyrine.

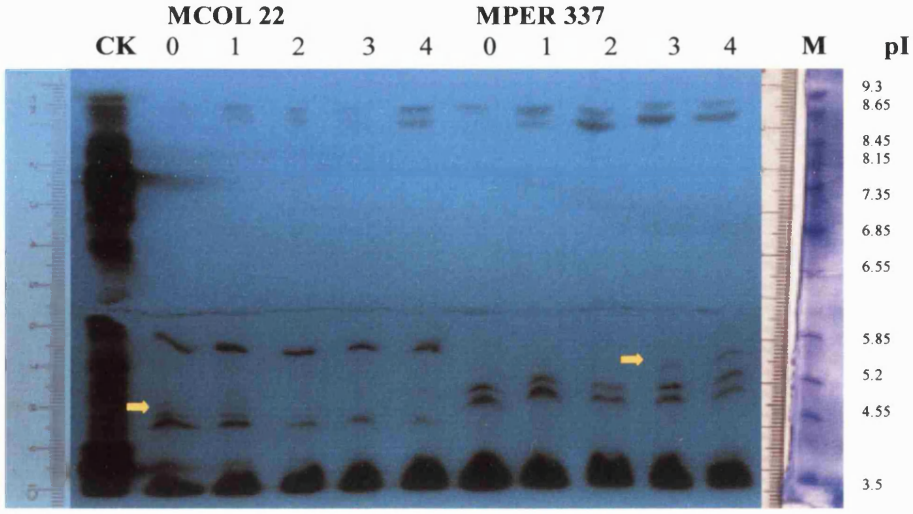


Figure 4.5.1 Guiacol staining of cassava storage root peroxidase isoforms run on an IEF PAG plate. CK= horseradish peroxidase 30μg, M= pI marker stained with Coomassie blue G. Total protein was extracted from roots at 0, 1, 2, 3, and 4 days after injury.

Using the guiacol staining method 2 cationic root peroxidase isoforms of pI > 8.45 were detected in both MCOL 22 and MPER 337. Since the bands migrated at equivalent distances they represent basic/cationic peroxidase isoforms present in both cultivars. Both cationic peroxidase bands showed an increase in staining intensity during the time course indicating they are up regulated during PPD, with the lower band first detected in both cultivars 1 day after injury.

The anionic peroxidases were more varied between the 2 cultivars, with MCOL 22 and MPER 337 showing different predominant anionic peroxidase isoforms. In MCOL 22 the 2 major anionic isoforms showed pIs of approximately 5.85 and 4.55, whilst in MPER 337 2 major activity bands were detected at pIs between 4.55 and 5.2. Such isozymes variation is commonly observed in other plant systems and has been used for cultivar identification (Hussein *et al.* 1987, Lefeire and Charner 1993a, b). The large diffuse bands at the anode baseline of the plate (pI 3.5) suggest that both cultivars contain several additional highly anionic peroxidases. Separation and visualisation of these highly anionic peroxidase isoforms would require electrophoresis on a narrower range anionic low pI plate such as Pharmalyte 2.5 – 5 (Amersham Pharmacia).

Changes were observed in the electrophoretic pattern of the anionic peroxidases of both cultivars. In MCOL 22 the isoform band of approximate pI 5.85 showed little change during the storage period, whilst the lower predominant activity band of pI just below 4.55 showed a decrease in staining intensity. In addition, a fainter band at around pI 4.55 (indicated by an arrow in figure 4.5.1) which was evident immediately after injury was no longer detected after 2 days. In MPER 337 the 2 main anionic peroxidase bands showed little change during the storage period, however a new isoforms of pI just below 5.85 (again indicated by an arrow in figure 4.5.1) appeared on days 3 and 4.

As noted in previous studies these data indicate the presence of a complement of peroxidase isoforms in the cassava storage root with differing expression patterns, perhaps reflected different physiological roles during PPD.

4.6 Tissue localisation of peroxidase expression

Transcript accumulation of MecPX1 in roots, petiole and leaves of the cassava plant was examined by northern blotting as described in section 2.7.10. Total RNA was extracted from plants of cultivar MDOM5 at CIAT, Colombia. Root samples were taken on the day of harvest. Hybridisation with MecPX1 showed a transcript of approximately 1.4kb with predominant expression in roots, and little or no expression detected in petioles or leaves as shown below (figure 4.6.1).

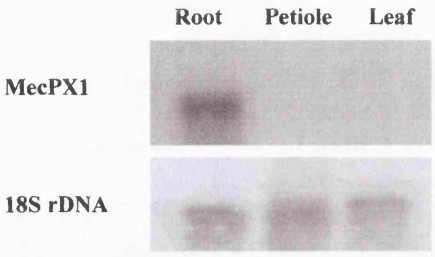


Figure 4.6.1. mRNA expression of MecPX1 in different tissues of the cassava plant. As a control for equivalent loading the blot was stripped and re-probed with a cDNA encoding cassava 18S rRNA (lower panel).

In order to examine overall peroxidase activity at the level of enzyme activity, tissue printing and light microscopy techniques utilising the peroxidase substrate guiacol were carried out as described in section 2.7.14. Tissue printing was carried out essentially according to Varner (1992). The detection method used is that of Peyrado *et al.* (1996). Peroxidase activity is indicated by the formation of a brown oxidation product. An example of results for cultivar MCOL 22 immediately after harvest (non deteriorated root), and at 5 days after harvest (deteriorated root) are shown below (figure 4.6.2).

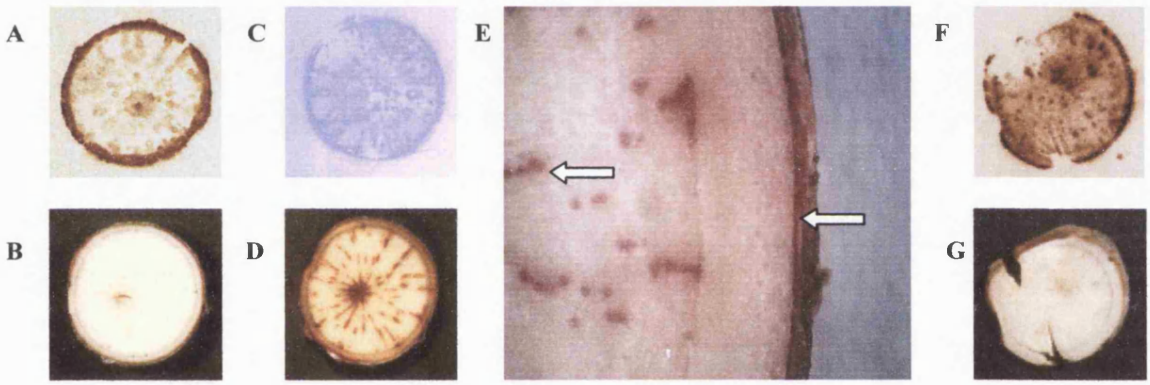


Figure 4.6.2 Tissue print detection and localisation of peroxidase activity in cassava roots. Panel A = tissue print on nitrocellulose paper showing peroxidase activity in a transverse slice of a non deteriorated root (T = 0 days). Panel B = tissue slice from which the tissue print was prepared. Panel C = tissue print prepared from the root slice shown in B and stained with Coomassie blue. Panel D = tissue slice from B stained with phluroglucinol. Lignified and suberised tissues stain red. Panel E = phloroglucinol staining of a transverse root slice viewed under a dissecting microscope (10X magnification). Phloroglucinol positive areas at the junction between the epidermis and cortex, and xylem tissues are indicated by arrows. Panel F = peroxidase activity in deteriorated root. Panel G = tissue slice from which the tissue print was prepared.

As control reactions, tissue print assays were carried out using heat treated tissue. These showed no colour development. In addition, total protein activity for tissue prints was detected by the Coomassie blue method of Mas and Pallas (1995) as described in section 2.7.14.4. These showed transfer of protein to the nitrocelluse paper from all parts of the root slices. Results for tissue printing experiments (figure 4.6.2) indicate

initial stages after harvest, peroxidase activity is localised to the epidermis, cortex and xylem tissues of the storage root, with little activity detected in the root parenchyma. Areas of peroxidase activity corresponded well with phloroglucinol positive areas. In the deteriorated root, peroxidase activity was extensive and had spread throughout the root parenchyma.

Peroxidase activity at the cellular level was examined by light microscopy as described in section 2.7.14.4. Hand cut sections of storage roots of cultivar MCOL 22 were examined immediately after harvest and again after 5 days storage. Examples of localisation experiments for non deteriorated and deteriorated roots are shown in figures 4.6.3 and 4.6.4 respectively.

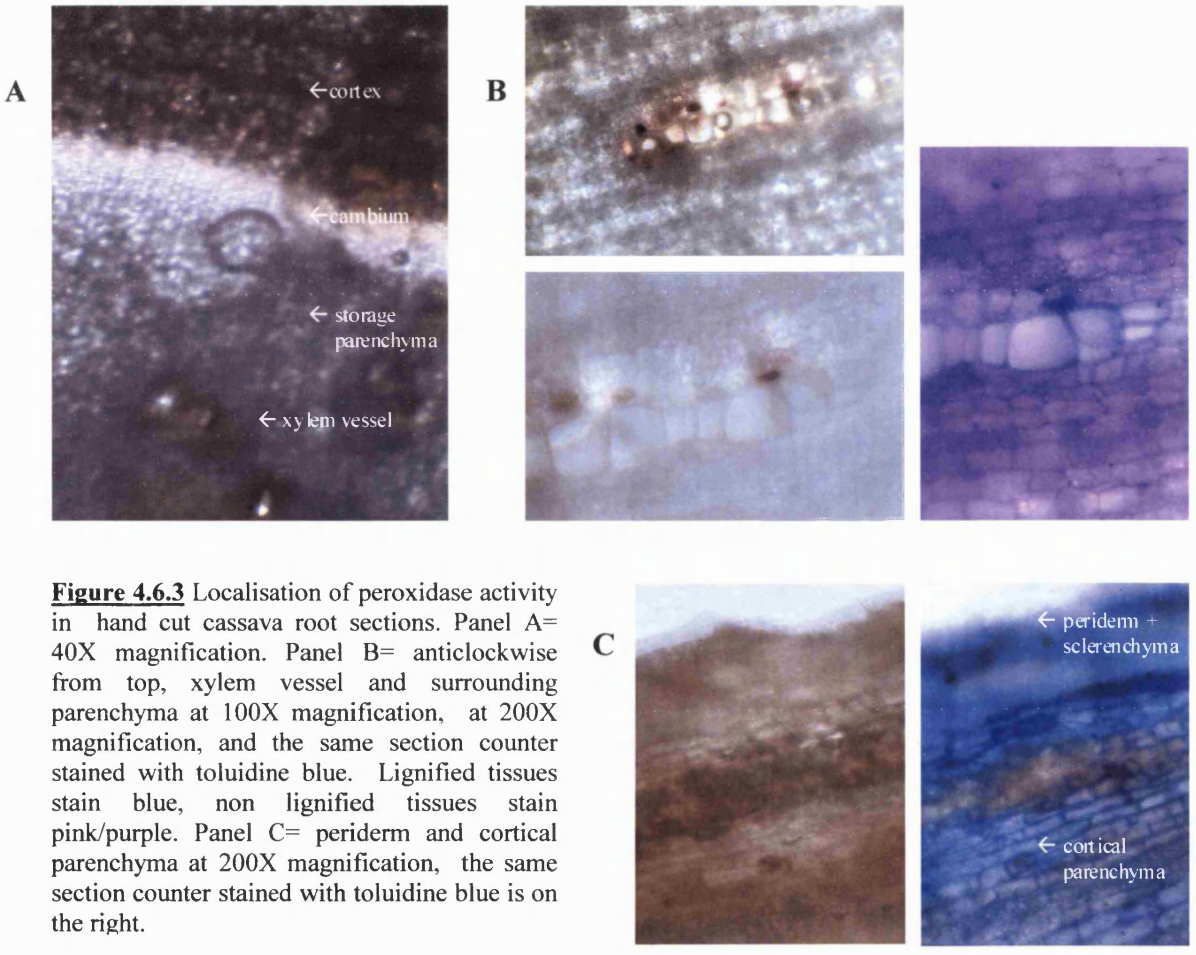


Figure 4.6.3 Localisation of peroxidase activity in hand cut cassava root sections. Panel A= 40X magnification. Panel B= anticlockwise from top, xylem vessel and surrounding parenchyma at 100X magnification, at 200X magnification, and the same section counter stained with toluidine blue. Lignified tissues stain blue, non lignified tissues stain pink/purple. Panel C= periderm and cortical parenchyma at 200X magnification, the same section counter stained with toluidine blue is on the right.

In freshly harvested root samples examined under low power (40X magnification, panel A figure 4.6.3) peroxidase activity again showed localisation to the tissues of the cortex and to the xylem vessels. No peroxidase activity as indicated by brown staining with the guiacol reagent was observed in the tissues of the intervening cambium or the storage parenchyma. When xylem vessels were examined at higher magnification (panel B) strong staining was observed in some of the parenchyma cells directly adjoining the xylem vessels, but not in the xylem vessels themselves. Staining of these neighbouring cells was diffuse throughout the cell, rather than associated with the cell wall. Since peroxidases are primarily targeted to either the cell wall or cell vacuole, this suggests that the predominant peroxidase isoforms active in the cassava root parenchyma immediately after harvest are vacuolar. The outer part of the cassava storage root consists of a periderm and a lignified sclerenchyma layer surrounding the cortical parenchyma which contains phloem cells (for review see Hunt *et al.* 1977). When this region was examined under higher magnification (panel C) strong peroxidase activity was again observed, particularly in the cortical parenchyma. Staining was intense and appeared to be both cell wall associated and vacuolar in certain cells.

Microscopic observations of deteriorated cassava root samples are shown in figure 4.6.4. below.

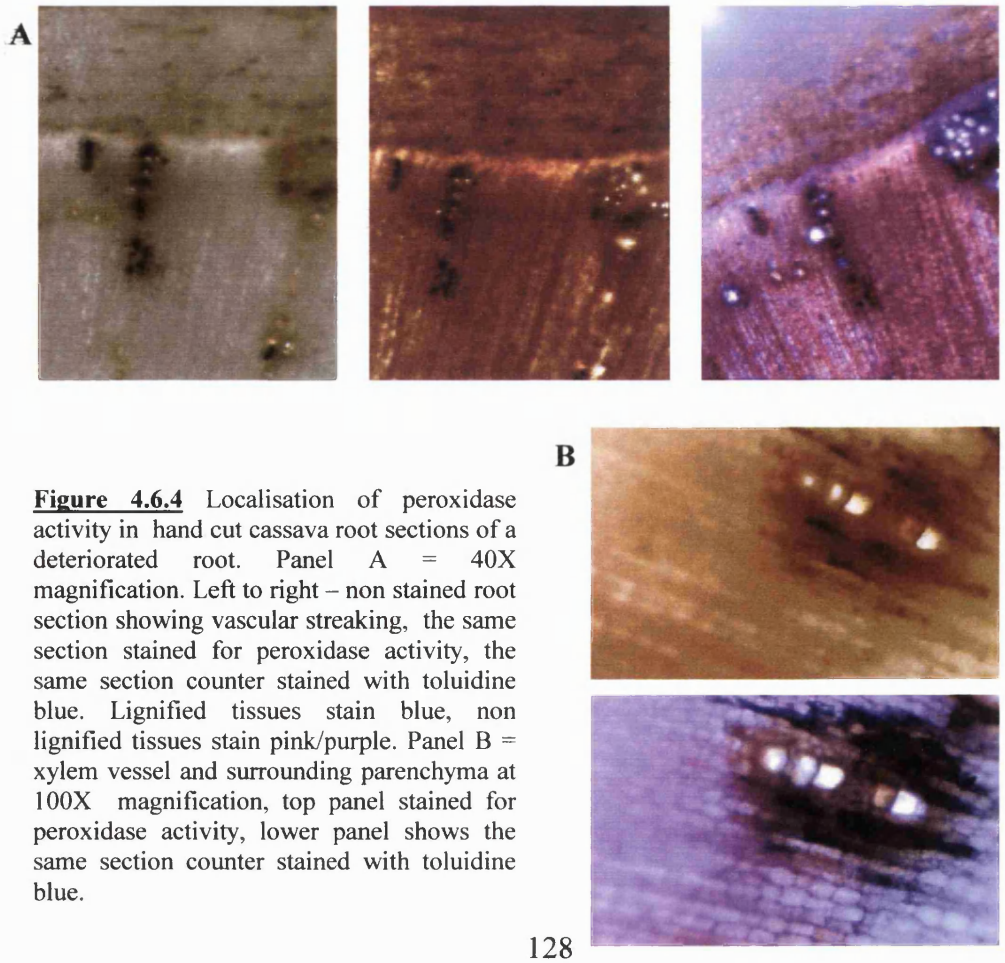


Figure 4.6.4 Localisation of peroxidase activity in hand cut cassava root sections of a deteriorated root. Panel A = 40X magnification. Left to right – non stained root section showing vascular streaking, the same section stained for peroxidase activity, the same section counter stained with toluidine blue. Lignified tissues stain blue, non lignified tissues stain pink/purple. Panel B = xylem vessel and surrounding parenchyma at 100X magnification, top panel stained for peroxidase activity, lower panel shows the same section counter stained with toluidine blue.

In deteriorated root samples viewed under low power (40X magnification, panel A figure 4.6.4) vascular streaking was clearly evident when non stained sections were viewed by light microscopy. As noted by Rickard (1983), vascular streaking was microscopically observed as amber brown/ green brown occlusions of the xylem vessels rather than the blue/ black colour which is observed macroscopically. Staining of tissue sections for peroxidase activity showed intense brown staining throughout the cortex and parenchyma as had been observed in tissue printing experiments. When xylem vessels and surrounding parenchyma tissue was examined at high power, brownish pigmented material was often observed in parenchyma cells adjacent to the xylem vessels. When stained with the guiacol reagent, peroxidase activity was again evident throughout the parenchyma. In several instances darker staining was observed surrounding the xylem vessels. Pigmented material in the xylem parenchyma and a xylem vessel showing granular occluding material as has been described by Rickard are shown in panel B (figure 4.6.4).

4.7 Effect of pruning treatment, ethylene and jasmonic acid on peroxidase transcript accumulation

Transcript accumulation of MecPX1 following pre-harvest pruning, and in response to treatment with ethephon and methyl jasmonate was examined by Northern blotting as described in section 2.7.10. Treatments were carried out as described in section 2.7.10.8. Total RNA was extracted from storage roots of cultivar MCOL 22 at CIAT, Colombia. Root samples were taken on the day of harvest. Attempts were made to extract RNA from root samples treated with the plant signalling molecule salicylic acid, which has been implicated in induction of defence related genes in several plant systems (Murphy *et al.* 1999, Scott *et al.* 1999, Yu *et al.* 1997). However, good quality RNA was not obtained after two attempts from either control or treated samples and this experiment was not continued.

For the pre-harvest pruning treatment, roots were obtained from plants which had been pruned by removal of the stem and all leaves approximately 30cm from the ground 2 weeks prior to harvest. Control RNA samples were prepared from similar non pruned plants harvested at the same time. For the ethylene treatment, root slices were incubated in the ethylene generating compound ethephon (Sigma) (0.02% in sterile water) for 24 hours in the dark. Control slices were incubated in water alone. For the methyl jasmonate treatment, root slices were incubated for 24 hours in the dark in methyl

jasmonate (Sigma) (500μM in 0.1% ethanol). As noted by Plumbley *et al.* (1981) accelerated deterioration was observed in root slices that had been treated with ethylene. Hybridisation with MecPX1 showed a transcript of approximately 1.4kb. The transcript was strongly induced by ethylene treatment. However, pre-harvest pruning, and treatment with methyl jasmonate had little or no effect on transcript accumulation (figure 4.7. 1 Panels B, A and C respectively).

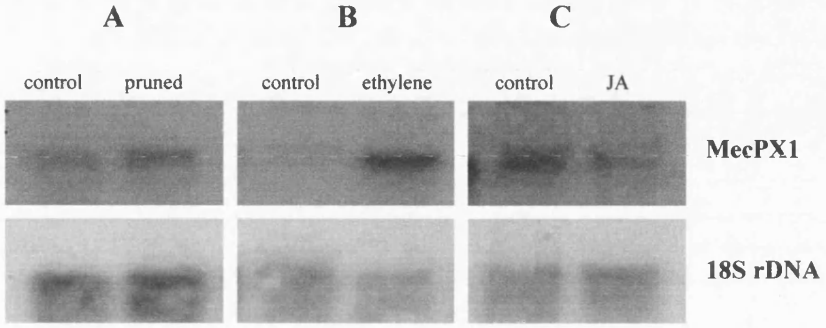


Figure 4.7.1 mRNA expression of cassava peroxidase MecPX1 in response to different treatments. For all treatment panels control samples are shown on the left, experimental treatment samples are shown on the right. Panel A = Pre harvest pruning treatment. Panel B = Ethephon treatment. Panel C = Jasmonic acid treatment.

4.8 Comparative peroxidase expression in different cultivars under storage conditions at the University of Bath.

Peroxidase transcript accumulation for MecPX1 and overall peroxidase enzyme activity during the post harvest period was compared for a range of cultivars showing differing susceptibility to PPD. Cassava storage roots for these experiments were wax dipped immediately after harvest and air freighted from CIAT, Colombia. On arrival, post-harvest physiological deterioration was induced by removal of the proximal and distal ends of the roots and cutting of 2 "V" shaped incisions through the epidermis along the length of the root. The root ends were covered with parafilm and roots were stored at ambient temperature on a bench top.

For northern blotting experiments total RNA was extracted from storage roots of cultivars MCOL 22 and MNGA 2 over a 6 day timecourse. Cultivar MCOL 22 has been characterised as showing high susceptibility to PPD, whilst MNGA 2 has been characterised as showing intermediate to low susceptibility. For these experiments, visible symptoms of PPD occurred in the highly susceptible cultivar MCOL 22 by the 3rd day after wounding of the root and was extensive by day 6. In the less susceptible

cultivar MNGA 2, slight vascular streaking was observed on day 4, and remained less pronounced than in MCOL 22 at day 6. Northern blotting and hybridisation were carried out as described in section 2.7.10. Results are shown in figure 4.8.1. Hybridisation with MecPX1 showed a transcript of approximately 1.4kb. The transcript was strongly induced during the post harvest storage period in roots of both cultivars. However, MecPX1 mRNA accumulated earlier and to a higher level in the susceptible cultivar MCOL 22.

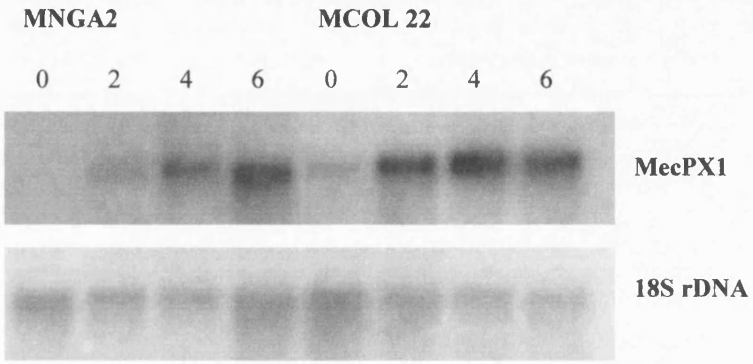


Figure 4.8.1 mRNA transcript accumulation of cassava peroxidase MecPX1 during the post harvest period. The time after harvest is indicated in days. 10μg aliquots of each sample were run on a denaturing formaldehyde gel and Northern blotted according to standard procedures (Sambrook *et al.* 1989). As a control for equal loading the same blot was stripped and re-hybridised with an 18S rDNA probe (lower panel).

Peroxidase expression at the level of enzyme activity was examined using the tissue printing method of Peyrado *et al.* (1996) as previously described in section 4.6. Although the technique provides qualitative rather than quantitative data, it does allow comparison of relative staining intensity and changes of localisation in different cultivars. Tissue prints were prepared from air freighted roots of cultivars MNGA 2, MCOL 22 and CM 21772. Cultivar MCOL 22 has been characterised as showing high susceptibility to PPD, whilst MNGA 2 has been characterised as showing lower susceptibility. Cultivar CM 21772 shows a highly variable PPD response and has been characterised as both high and low PPD response in different evaluation experiments carried out at CIAT (Teresa Sanchez pers. com). For all experiments carried out under University of Bath storage conditions however, roots of CM 21772 consistently showed

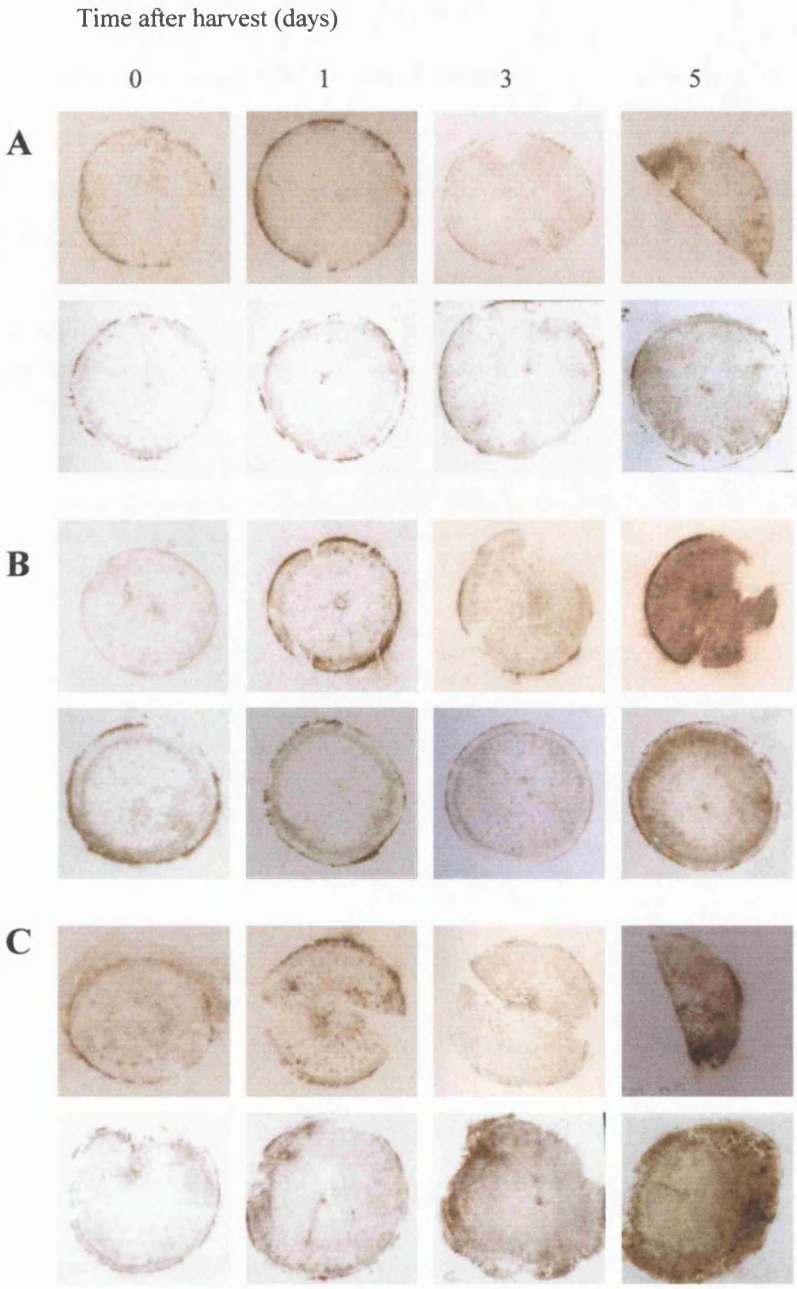


Figure 4.8.2. Tissue print detection and localisation of peroxidase activity in cassava storage roots during the post harvest period. Results of 2 experiments are shown in each panel. Panel A = cultivar MNGA 2 (less susceptible). Panel B = cultivar MCOL 22 (highly susceptible). Panel C = cultivar CM 21772 (highly susceptible)

very high susceptibility to PPD. For these experiments, visible symptoms of PPD occurred in cultivar CM 21773 (panel C, figure 4.8.2) at day 2 (upper panel) or day 1 (lower panel) after injury of the roots. For the highly susceptible cultivar MCOL 22, vascular streaking occurred by day 3 after wounding of the root and was extensive by day 5. In the less susceptible cultivar MNGA 2, slight vascular streaking was observed on day 4, and remained less pronounced than in MCOL 22 at day 6. Results of duplicate tissue printing experiments carried out over a time course of 5 days are shown in figure 4.8.2. All cultivars showed changes in localisation of peroxidase activity during the storage period, with initial activity in the cortex and vascular tissues spreading throughout the parenchyma as deterioration progressed. A common observation was that the spreading of activity through the root parenchyma occurred on the same day that symptoms of vascular streaking were first observed. In addition, the rapidly deteriorating cultivars CM 21772 and MCOL 22 showed earlier and more intense staining than the less susceptible cultivar MNGA 2. Similar initial experiments carried out using additional cultivars - SM 9859 (high PPD response), CM 7033 (low PPD response) and MBRA 337 (low PPD response) were in agreement with the observation of higher levels of peroxidase activity in storage roots of more highly susceptible cultivars.

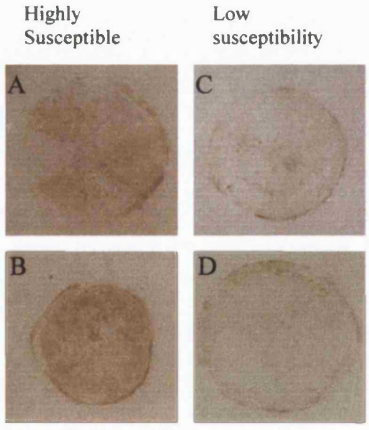


Figure 4.8.3. Tissue print detection and localisation of peroxidase activity in cassava storage roots during the post harvest period. All tissue prints were prepared from air freighted storage roots 2 days after injury. Panel A = cultivar MCOL 22 (highly susceptible). Panel B = cultivar SM 9859 (highly susceptible). Panel C = cultivar CM 7033 (low susceptibility). Panel D = cultivar MBRA 337 (low susceptibility).

4.9 Comparative peroxidase expression in different cultivars under field storage conditions at CIAT, Colombia

Peroxidase transcript accumulation for MecPX1 and overall peroxidase enzyme activity during the post harvest period under field storage conditions were compared for four cultivars showing differing susceptibility to PPD. Cassava storage roots for these experiments were freshly harvested from the field and were then injured by removal of

the proximal and distal ends of the roots and cutting of 2 "V" shaped incisions through the epidermis along the length of the root. The root ends were covered with parafilm and roots were stored in an open air shed. For northern blotting experiments total RNA was extracted from storage roots over a 5 day time course. Cultivars used were MCOL 22, MNGA 2, MDOM 5 and CM 21772. Under these storage conditions visible symptoms of PPD occurred in storage roots of all cultivars within 24 hours after harvest and the PPD response progressed rapidly. Rates of deterioration in MCOL 22, MNGA 2 and MDOM 5 were roughly similar, whilst CM 21772 showed slightly less pronounced deterioration. Northern blotting and hybridisation was carried out as described in section 2.7.10. Results are shown in figure 4.9.1. Hybridisation with MecPX1 again showed a transcript of approximately 1.4kb. The transcript was strongly induced during the initial post harvest storage period (0-3 days) in roots of all cultivars and declined thereafter. This decline was not observed under University of Bath storage conditions (section 4.8) and may be related to increased desiccation of the root tissues under field storage conditions in Colombia, and/ or to the more rapid progression of PPD under these conditions. Levels of MecPX1 transcript accumulation were roughly similar in roots of cultivars MCOL 22, MNGA 2 and MDOM 5, but were lower in the less rapidly deteriorating cultivar CM 21772.

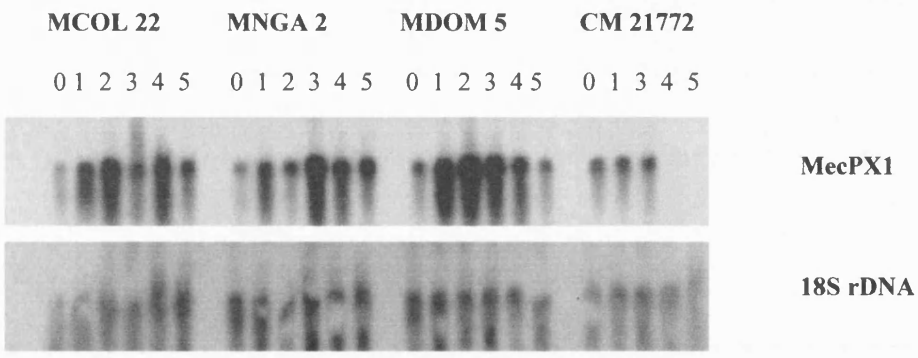


Figure 4.9.1 mRNA transcript accumulation of cassava peroxidase MecPX1 during the post harvest period. The time after harvest is indicated in days. 10μg aliquots of each sample were run on a denaturing formaldehyde gel and northern blotted according to standard procedures (Sambrook *et al.* 1989). As a control for equal loading the same blot was stripped and re-hybridised with an 18S rDNA probe (lower panel).

Peroxidase expression at the level of enzyme activity was again examined using the tissue printing method used of Peyrado *et al.* (1996). Roots of cultivars MCOL 22,

MNGA 2, MDOM 5 and CM 21772 were harvested and stored under field conditions as previously described. Tissue print experiments were carried out over a 4 day time course using 3 storage roots of each cultivar. Symptoms of vascular streaking again occurred within 24 hours after harvest. Rates and intensity of deterioration were similar in MCOL 22, MNGA 2 and MDOM 5, with CM 21772 showing less pronounced symptoms of deterioration. Results are shown in figure 4.9.2. Peroxidase activity had spread throughout the root parenchyma in all roots examined by day 2. Staining intensities were again somewhat lower in the more slowly deteriorating cultivar CM 21772. These data were in agreement with experiments carried out under Bath storage conditions and indicate that higher peroxidase activity is correlated with increased susceptibility to PPD.

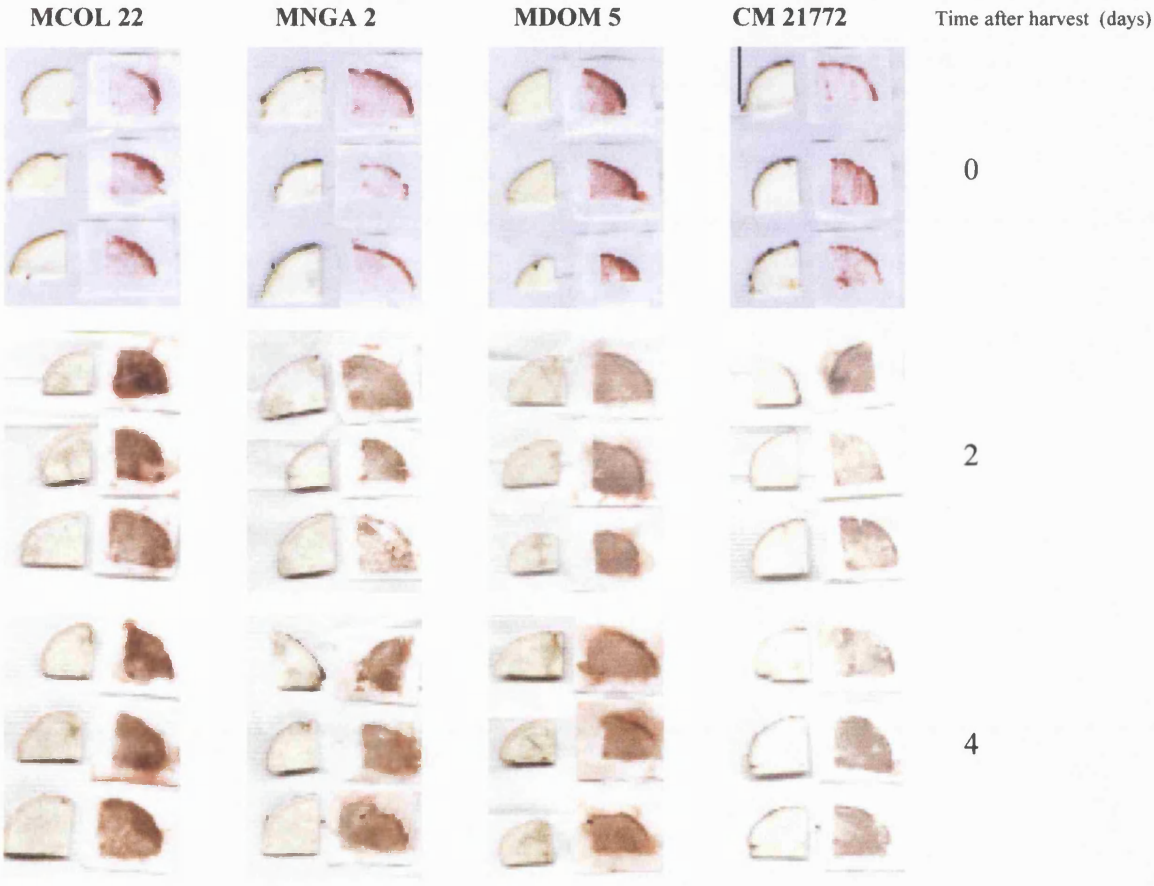


Figure 5.9.2. Tissue print detection and localisation of peroxidase activity in cassava storage roots during the post harvest period. Results of experiments on 3 roots of each cultivar stored under field conditions are shown in each panel. Tissue prints stained for peroxidase activity are shown on the right, tissue slices from which the prints were taken are shown on the left. Please note that the pink/ red colour of the prints at day 0 is because a different scanner was used for documentation at this time point.

4.10 Conclusions and discussion

Results presented here describe the isolation and characterisation of 2 partial peroxidase cDNA clones designated MecPX1 and MecPX2. The sequence and deduced translation of MecPX2 has been lodged with the Genbank database under the accession number AY033386 and the clones have been transferred to CIAT for inclusion on the cassava genetic map. Both clones were truncated at the 5' and 3' ends and were identical, although MecPX2 was a larger transcript and contained additional sequence at both the 5' and 3' ends. Sequence analysis indicate that the protein encoded by MecPX1 and MecPX2 is likely to be a cationic peroxidase and thus may be targeted to the vacuole in cassava storage roots (Campa 1991, Mader 1992, Brownleader 1995). Southern blotting experiments indicate the cognate gene may be present in the cassava genome in 2 copies and forms part of a gene family comprising at least 3 members. However, a low level of sequence similarity between plant peroxidases from the same plant has commonly been observed e.g. in spinach and *Arabidopsis* where similarity ranges from 52% to 92% (Simon *et al.* 1996). Thus the true number of cassava peroxidase genes may be considerably higher than detected under the stringency conditions used for Southern blotting. With regard to future isolation of additional cassava peroxidase sequences, the high degree of sequence divergence was a problem in this study. A perhaps better approach would be to use the primer PX-oligo described in this study - based on the conserved motif FHDCV within the catalytic and distal heme binding domain of plant peroxidases - for oligonucleotide library screening. This region is less strongly conserved in the ascorbate peroxidases, however a strongly conserved motif GRxD within conserved domain B of unknown function was fully conserved in both the secretory and ascorbate peroxidases (figure 4.3.2) and could be used as a basis for the design of additional primers.

Northern blotting experiments indicate that the peroxidase encoded by the isolated clones is expressed primarily in storage roots of the cassava plant, but not leaves or petioles, and is strongly up-regulated during PPD. Higher levels of transcript accumulation were detected in roots of susceptible cultivars relative to less susceptible cultivars in experiments carried out both at the University of Bath and under field conditions at CIAT, Colombia. The transcript was strongly induced by ethylene, but unaffected by pre-harvest pruning or methyl jasmonate treatment. Ethylene is associated with fruit ripening, floral senescence and abscission in other plant systems and is produced in cassava storage roots in response to injury after a short lag of around 6 hours. Several authors (Plumbley *et al.* 1981, Wheatley 1982) have suggested that this

increase in ethylene may affect root discoloration by altering peroxidase regulation. As discussed in the introduction, numerous studies have indicated that pre-harvest pruning 1 – 2 weeks before harvest can reduce the susceptibility of the cassava storage root to PPD (Tanaka *et al.* 1983, Tanaka *et al.* 1984, Data *et al.* 1984, Kato *et al.* 1991). This reduced susceptibility is often accompanied by changes in root composition and toughness suggesting increased lignification of the root prior to harvest. When peroxidase activity, as measured by enzyme assays, is compared in roots from pruned and non pruned plants these studies have indicated lower overall peroxidase activity in the roots from pruned plants.

At the level of protein expression, cassava root peroxidase isoforms expressed during the post harvest period were examined by IEF PAG in 2 cultivars over a 5 day time course. A larger number of anionic than cationic isoforms was observed. Since the IEF gel used was of a broad pI range (3.5 – 9.5) more than one highly anionic band ran together and the actual number of discrete anionic peroxidase isoforms may be higher. PAGE studies by Plumbley and Hughes (1982) and Padmaja and Balagopal (1985) had indicated the presence of 6 or 7 peroxidase isozymes respectively in non deteriorated root, whilst 7 (or 8) were detected in deteriorated root (22-24 hours after injury). In this study, 5 isoforms were detected in MCOL 22 immediately after injury, with 4 isoforms detected in MPER 337. By 24 hours after injury, 6 and 5 isoforms were detected in MCOL 22 and MPER 337 respectively. Peroxidase isoform bands showed changes in activity during the post harvest period, with some bands showing increased activity, others showing decreased activity and others remaining unchanged. Although results from IEF PAG and PAGE can not be compared directly since during IEF PAG isoforms separate on the basis of pI rather than molecular size, these results were essentially in agreement with earlier studies (Marriot *et al.* 1981, Plumbley *et al.* 1981) and indicate that the cassava storage root contains a complement of peroxidase isoenzymes which show differing regulation during PPD reflecting different physiological roles. Both cationic isoforms detected here showed up regulation during the post harvest period. This expression pattern was similar to the profile of MecPX1 transcript accumulation during post harvest storage, and it is probable, although not conclusive, that the isolated cDNA clones MecPX1 and MecPX2 correspond to one of the cationic activity bands detected by IEF PAG. In the study of Plumbley *et al.* (1981) a single peroxidase isozyme (Rf 0.3) showed up regulation in response to ethylene treatment and was strongly up regulated during deterioration. Since a similar expression profile was detected for the MecPX1 transcript, at least at the level of transcription, it is tempting to

speculate that the peroxidase encoded by MecPX1 corresponds to the R_f 0.3 isozyme observed in previous studies.

Further studies which could allow putative allocation of the roles played by the isoforms detected here would be of considerable interest. Whilst several studies have suggested that acidic isoforms are primarily involved in lignification (Brownleader 1995, Boeuf *et al.* 2000), several recent studies have proposed a role for basic peroxidases (Liu and Ger 1997, Wallace and Fry 1999, Quiroga *et al.* 2000). The colorimetric substrate guiacol, used for staining of IEF PAG gels in this study, allows detection of all peroxidase isoforms. In contrast, the substrate syringaldehyde (an analogue of lignin monomer), is oxidised by specific isoforms and reactivity towards syringaldehyde has been used to assign a role in lignification (Peyrado *et al.* 1996, Boeff *et al.* 2000, Quiroga *et al.* 2000). In addition, the peroxidase substrate scopoletin, may be of particular interest regarding PPD of cassava roots. The naturally occurring coumarin, scopoletin, shows a transient accumulation in cassava roots peaking within 10-24 hours after injury (Tanaka *et al.* 1983, Buschman pers. com.), and has been proposed as the substrate for the deterioration response (Uritani *et al.* 1984, Wheatley 1980). Initial experiments carried out in this laboratory (Gomez Vasquez pers. com.) have indicated the presence of cassava root peroxidase isoforms with activity towards scopoletin. Thus, a comparison of IEF PAG gels stained by guiacol based, syringaldehyde based, and scopoletin based staining methods, could enable tentative identification of the roles played by the various cassava root peroxidase isoforms during PPD.

Cytochemical and histochemical approaches were used to examine overall peroxidase enzyme activity at the tissue and cellular level. Although these approaches give qualitative data they allow detection of small localised changes at the tissue (histochemistry) or cellular (cytochemistry) level, and thus provide complementary information to molecular or biochemical analyses which are global in nature and thus mask the heterogeneity and compartmentalisation found in plant tissues. At the histochemical level, a tissue printing approach was used, and indicated that a change in localisation of peroxidase activity occurs during PPD. In the initial stages after harvest, peroxidase activity is largely confined to lignified tissues of the root in the cortex and xylem tissues. As the PPD response progresses, activity is detected throughout the root. This change in localisation could reflect induction or up-regulation of certain peroxidase isoforms, and/ or could reflect increased "leakiness" of cell compartments during PPD as a result of membrane damage. When peroxidase activity staining intensities were compared in cultivars showing differing PPD responses, greater staining intensity,

indicating higher levels of peroxidase activity, was observed in susceptible cultivars. These data confirmed and expanded previous work showing increased peroxidase activity as measured by enzyme assays during the course of PPD (Marriott *et al.* 1981, Rickard and Gahan 1983, Tanaka *et al.* 1983, Uritani *et al.* 1983), and reports of higher levels of peroxidase activity in roots of more susceptible cultivars (Campos and Carvallho 1990). Cytochemical observations using light microscopy confirmed the change in peroxidase localisation observed by tissue printing and allowed more accurate localisation to particular tissues and cells. In the initial stages after harvest, peroxidase activity was again observed predominantly in the cortex and in the region of xylem tissues. Activity was localised to particular cells of the xylem parenchyma directly adjacent to the xylem vessels and appeared to be dispersed throughout these cells rather than associated with the cell walls, suggesting a vacuolar location. This localisation is of particular interest given the cytological studies by Rickard (1983) indicating the initial site of production of the coloured occlusions associated with PPD in the xylem parenchyma cells, followed by movement into the xylem vessels via pit areas.

CHAPTER FIVE:
STUDIES ON THE ROLE OF SUPEROXIDE DISMUTASE DURING POST-HARVEST PHYSIOLOGICAL DETERIORATION

5.1 Introduction and literature review

5.1.1 Types and classification of plant superoxide dismutase

The enzyme superoxide dismutase (superoxide:superoxide oxidoreductase, EC 1.15.15) catalyses the dismutation of superoxide (O_2^-) to hydrogen peroxide and oxygen, and is one of the primary reactive oxygen species (ROS) scavenging enzymes found in aerobic organisms. In plants, three types of superoxide dismutase (SOD) are distinguished on the basis of the metal cofactor at the active site - manganese superoxide dismutase (MnSOD), Copper/Zinc superoxide dismutase (Cu/ZnSOD) and iron superoxide dismutase (FeSOD). FeSOD is found in prokaryotes, but not in yeast or vertebrates, and it has been proposed that plants acquired FeSOD through an endosymbiont ancestor of chloroplasts, and subsequent gene transfer from the chloroplast to the nucleus (Van Camp *et al.* 1990). MnSOD and FeSOD show considerable sequence similarity and in some instances the apo-enzymes may function with either metal present at the active site, whilst CuZnSOD represents a distinct structural class that is found almost exclusively in eukaryotes (Bowler *et al.* 1989, Scandalios 1997). The 3 classes of SOD may be distinguished by their sensitivity to inhibition by H_2O_2 and KCN, since FeSOD is resistant to both, FeSOD is sensitive to H_2O_2 only and CuZnSOD is sensitive to both (Manchenko 1994).

Plants are the only group to contain all 3 classes of SOD, which may be encoded by a single gene or a small gene family for each class. In higher plants, 2 forms of CuZnSOD have been found, one occurring in the cytosol the other in the chloroplasts. In tobacco, 4 SOD isozymes have been described and are encoded by 2 MnSOD gene family members (SodA1 and SodA2), a single FeSOD (SodB), and a single CuZnSOD (SodCp) (Van Camp *et al.* 1997). Similarly, in rubber (*Hevea brasiliensis*) 2 MnSOD family members are present (Miao and Gaynor 1993). In maize (*Zea mays*) there are at least 9 distinct SOD genes. MnSODs are encoded by a small gene family comprising at least 4 members, and 5 Cu/ZnSOD isozymes – 4 cytosolic and 1 chloroplast associated - are encode by 5 similar but not identical genes (Van Camp *et al.* 1994, Scandalios 1997).

FeSOD and CuZnSODs occur as dimers, whilst MnSOD may occur as a dimer or tetramer. Each class may occur as one or more isozymes, although the number of isozymes varies between different plants (Bowler *et al.* 1994). For example 4 SOD isozymes have been reported in Mexican red bean (Milosovic and Slusarenko 1996) whilst in maize, 4 or 5 SOD isozymes have been detected by polyacrylamide gel

electrophoresis depending on the inbred line examined (Scandalios 1990, Scandalios 1997).

5.1.2 Location and targeting of plant superoxide dismutase

SOD activity in plant systems is primarily detected in the cytosol, chloroplasts, and mitochondria. In well studied plant systems such as tobacco and maize, the 3 classes of SOD isoform are nuclear encoded, but are localised to different sub cellular compartments (Van Camp *et al.* 1997). MnSOD occurs in the mitochondria, FeSOD in the chloroplasts and CuZnSOD in both the cytosol and chloroplasts. In some plant systems, peroxisomal and extracellular SOD activities have been reported (Bowler *et al.* 1994, del Rio *et al.* 1998, Scandalios 1997).

A characteristic transit peptide for mitochondrial proteins – rich in positively charged and neutral amino acids, and containing a core consensus motif Ser/Thr-Leu-Pro-Asp-Leu occurs at the amino terminal end of plant and mammalian MnSODs and is believed to direct transport to the mitochondria (Miao and Gaynor 1993, Bowler *et al.* 1989). Both FeSOD and CuZnSOD deduced polypeptides contain putative N-terminal sequences for chloroplast targeting. Cu/ZnSOD often occurs as two or more isoforms, one of which is located in the cytosol whilst the other is targeted to chloroplasts (Bowler *et al.* 1989). Cognate polypeptides show a high degree of sequence similarity apart from a short stretches of amino acids specific for chloroplastic or cytosolic types. Cu/ZnSOD activity has also been reported in peroxisomes (del Rio *et al.* 1998, Kleibenstein *et al.* 1998). Putative PTS1 type carboxy-terminal targeting signal motifs (Cys/Ala/Ser/Pro-Lys/Arg- Ile/Leu/Met) found in other plant peroxisomal proteins such as isocitrate lyase, malate synthase and uricase (Hayashi *et al.* 1997) have been noted in a number of plant CuZnSOD deduced polypeptides (Kliebenstein *et al.* 1998).

5.1.3 Properties and reactions catalysed by plant superoxide dismutase

Dismutation of O_2^- may occur spontaneously or catalysed by SOD according to the equation:

$$2\,O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

The rate of the spontaneous reaction is $k_2 \sim 8 \times 10^5\ M^{-1}\ s^{-1}$, whilst that of the SOD catalysed reaction is $k_2 \sim 0.2\ M^{-1}\ s^{-1}$ and the enzyme is able to react with O_2^- at rates limited only by diffusion (Scandalios 1997). The primary function of SOD, as well as

catalase, is to rapidly remove O_2^- and H_2O_2 respectively, thereby preventing formation of the highly reactive hydroxy radical ($OH^\bullet$) via the iron catalysed Haber-Weiss reaction:

$$O_2^{\bullet -} + H_2O_2 \rightarrow OH^\bullet + OH^- + O_2$$

The catalytic mechanism of all 3 SOD classes found in plants is thought to be similar and involves the attraction of O_2^- radicals in to the active site due to the presence of a positively charged protein pocket at the active site. The transition metal at the active site then carries out a one electron transfer between two radicals and undergoes alternating oxidation/reduction reactions (Bowler *et al.* 1994).

5.1.4 Functions of plant superoxide dismutases

Since the three SOD classes show differing subcellular localisation they might be expected to play slightly different physiological roles. A complex pattern of differential regulation of isoforms has been reported in different tissues, during development and in response to environmental stimuli. In tobacco, FeSOD is abundant in leaves, whilst CuZnSOD is detected only in immature leaves. Thus scavenging of O_2^-produced during photosynthesis in maize is believed to be effected primarily by FeSOD. The two MnSODs *SodA1* and *SodA2* are highly similar at the amino acid level, however their cognate gene promotor regions are very divergent and the two isoforms are expressed in different tissues (Van Camp *et al.* 1997). In maize, significant differences have been noted in the promoter regions of 2 cytoplasmic SOD genes – *Sod4A* and *Sod4* - which encode highly similar proteins. For example, *Sod4A* lacks an abscisic acid (ABA) response element found in *Sod4*, and is unresponsive to ABA treatment (Scandalios 1997). Differential regulation of SOD isoforms in response to metabolic and ozone stress, sugar concentration, ethylene, absicisic acid (ABA), salicylic acid (SA), wounding, fungal infection and herbicides such as paraquat have been reported (Bowler *et al.* 1989, Roa *et al.* 1997, Pitcher and Zilinskas 1996, Scandalios 1997).

No previous studies have been carried out on the role of SOD during PPD of cassava storage roots, however during the course of this project a cytosolic CuZnSOD was isolated from a cassava cell culture cDNA library (Lee *et al.* 1999).

5.1.5 Chapter summary

The generation of a probe corresponding to a cassava cytosolic CuZnSOD is described. Superoxide dismutase enzyme activity and isoform pattern during the post-harvest period was examined by polyacrylamide gel electrophoresis. The isolated cDNA probe was used to examine expression of cytosolic CuZnSOD in different tissues and in response to pre-harvest pruning, ethylene and methyl jasmonate treatment. Expression of the transcript during post-harvest storage was examined in a range of cultivars showing differing susceptibility to PPD.

5.2 Superoxide dismutase cDNAs expressed during PPD of cassava storage roots

In order to generate cassava specific SOD probes for screening of the PPD related cDNA library, a PCR approach similar to that described in section 4.2 was used. For CuZnSOD a primer designated oligoCuZnSOD was designed, based on a highly conserved sequence at the 3' end of known CuZnSODs. The primer has the sequence 5'-GATGAICTTGGIAAGGGICATG-3' and contains deoxyinosines at ambiguous nucleotide positions. For MnSODs a primer designated oligoMnSOD, with the sequence 5'-CCITAIGAITAIGGIGCICTIGAICCIGC-3' which had previously been used to isolate a tobacco MnSOD (Bowler *et al.* 1989) was used.

Each primer was used to carry out 2 PCR reactions using a 5µl aliquot of the cassava root cDNA library as template DNA. For PCR reaction 1 oligoCuZnSOD (or oligoMnSOD) was used in combination with the λgt10 forward primer (New England Biolabs); for PCR reaction 2 it was used in combination with the λgt10 reverse primer. Since the library is not directional this would allow amplification of cDNA inserts which may be ligated into the λgt10 vector in either orientation. Results of the PCR amplification reactions are shown in figure 5.2.1.

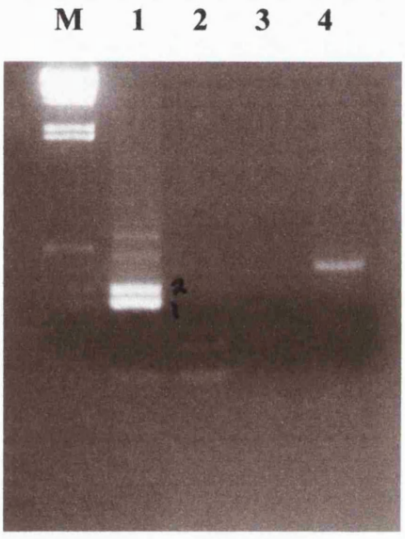


Figure 5.2.1 Cassava superoxide dismutase PCR products separated on a 2% TAE gel Lane M = marker DNA. Lane 1 = CuZnSOD PCR reaction 1 (oligo CuZnSOD + λgt10 forward primers). Lane 2 = CuZnSOD PCR reaction 2 (oligoCuZnSOD + λgt10 reverse primers). Lane 3 = MnSOD PCR reaction 1 (oligoMnSOD + λgt10 forward primers). Lane 4 = MnSOD PCR reaction 2 (oligoMnSOD + λgt10 reverse primers).

The CuZnSOD PCR amplification reactions (figure 5.2.1, lanes 1 and 2) generated a number of faint bands for both forward and reverse amplification reactions; but 2 strong bands of approximately 350bp were obtained with the oligoCuZnSOD and λgt10 foward primer combination. The MnSOD PCR amplifications generated a single band only, of approximately 400bp, using the oligoMnSOD and λgt10 reverse primer combination (figure 5.2.1, lane 4). The largest of the CuZnSOD bands and the MnSOD band were excised from the gel, purified as described in section 2.7.3, and were sequenced on an ABI 337 automated fluorescent sequencer. Sequence data was submitted to a blastx search using the NCBI database (www.ncbi.nlm.nih.gov). The oligoMnSOD PCR amplification product showed no homology to any known sequences on the database; however the CuZnSOD amplification product was confirmed as showing a high degree of homology to the the 3' region of plant cytoplasmic CuZnSODs. The predicted polypeptide showed highest similarity to a papaya (*Carica papaya*) cytosolic CuZnSOD sequence. At this time the full length sequence of a cassava cytosolic CuZnSOD (designated mSOD1) was published by Lee and colleagues (1999), and was assumed to be identical to the PCR product presented here, since the deduced polypeptide of Lee *et al.* showed only 3 amino acid differences to the corresponding region in the CuZnSOD PCR product. For this reason the PCR product was sequenced along both stands as shown in figure 5.2.2 and was used directly as a probe for downstream Southern and northern blotting applications. The nucleotide and deduced amino acid sequences of the CuZnSOD amplification product (MecCuZnSOD-PCR), corresponding to the 3' coding region and 3' UTR of plant cytosolic CuZnSODs is shown in figure 5.2.3.

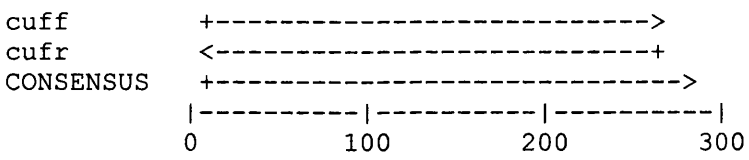


Figure 5.2.2 Diagrammatic representation of the sequencing strategy used for sequencing of MecCuZnSOD-PCR. The λgt10 forward primer and the oligoCuZnSOD primer which had been used for original amplification of the PCR product were used to generate sequence data for both strands.

```
1     gcttgggaaggggggcatgaacttagcaaaaccactggaaatgctggtggcagggtagc
        L  G  K  G  G  H  E  L  S  K  T  T  G  N  A  G  G  R  V
61    atgtggtgttattggtttgcaaggatagaatgattccccagggattcatgataaggcgaa
      A  C  G  V  I  G  L  Q  G
121   ggcagctgaataatgtattagctggaaattttaggcgaacgttgcaagcaaagaacaaaa
181   tcgtaattaaacttctggctggtttgccccgtttgtttttgtgatggaaaatgttgtgtg
241   cc
```

Figure 5.2.3 Consensus sequence of the cassava CuZnSOD amplification product MecCuZnSOD-PCR. The amber stop codon (tag) and a putative polyadenylation signal (aattaaa) are shown in bold. Amino acid residues within the predicted polypeptide that differ from the sequence of Lee *et al.* 1999 are shown in blue.

The MecCuZnSOD-PCR sequence was initially thought to be identical to the mSOD1 sequence of Lee *et al.* (1999) on the basis of amino acid comparison in the coding region. However a more careful examination at the nucleotide sequence level indicated the sequences differed substantially, particularly in the 3' UTR as shown in figure 5.2.4. Whilst a difference of 2-3 amino acid residues might be expected in a PCR product, and could be due to errors introduced during PCR, the level of difference between the sequences is higher than would be expected if MecCuZnSOD-PCR was a PCR product corresponding to the mSOD1. The quality of sequence data generated for sequencing of MecCuZnSOD-PCR was good (see appendix B), with a single ambiguity only occurring in the consensus sequence generated with the gcg GELSTART compiling programme. The MecCuZnSOD-PCR sequence therefore corresponds to a second cytosolic CuZnSOD in cassava.

```
1     gcttgggaaggggggcatgaacttagcaaaaccactggaaatgctggtggcagggtagc
425   tcttggcaggggaggacatgaactcagtaaaaccaccggaaatgctggtggcagagtagc

61    atgtggtgttattggtttgcaaggatagaatgattccccagggattcatgataaggcgaa
485   atgcggtattattggtttgcgaggatagagtgcttctccagagatcaataacaagacaaa

121   ggcagctgaataatgtattagctggaaattttaggcgaacgttgcaagcaaagaacaaaa
545   gacagctgaaacatgcacagccggacaacctttagaagaacgttaggagaccattaactc

181   tcgtaattaaacttctggctggtttgccccgtttgtttttgtgatggaaaatgttgtgtg
645   atttgaataaaagaaagaataatactgtagttttggctggtttggtcttgtgatctcaag

241   cc
665   at
```

Figure 5.2.4 Comparison of nucleotide sequences of MecCuZnSOD-PCR and the corresponding region of cassava mSOD2 of Lee *et al.* 1999. The MecCuZnSOD-PCR sequence is shown in black, the sequence of Lee *et al.* is shown in blue. Regions of sequence that differ are highlighted in grey. The stop codon (tag) and a putative polyadenylation signal (aattaaa) for both sequences are shown in bold.

5.3 Superoxide dismutase gene organisation in cassava

Southern blot hybridisations were carried out using MecCuZnSOD-PCR as a probe. Hybridisations were carried out overnight at 55°C as described in section 2.7.9. For the low stringency wash membranes were washed twice for 20 minutes in 2X SSC, 0.1% SDS at 55°C. For the high stringency wash, the membranes were washed for 20 minutes in 0.1X SSC, 0.1% SDS at 60°C. For the MecCuZnSOD-PCR probe (of size 423bp) the minimum percentage homology required to allow stable probe-target hybridisation under low and high stringency conditions were 76% and 93% respectively, as calculated using the equation $Tm°C = 81.5°C + 16.6\log [Na^+] + 0.41(\%GC) - (600/1)$ as described in section 2.7.9 and assuming a GC content of 50%. Results are shown in figure 5.3.1.

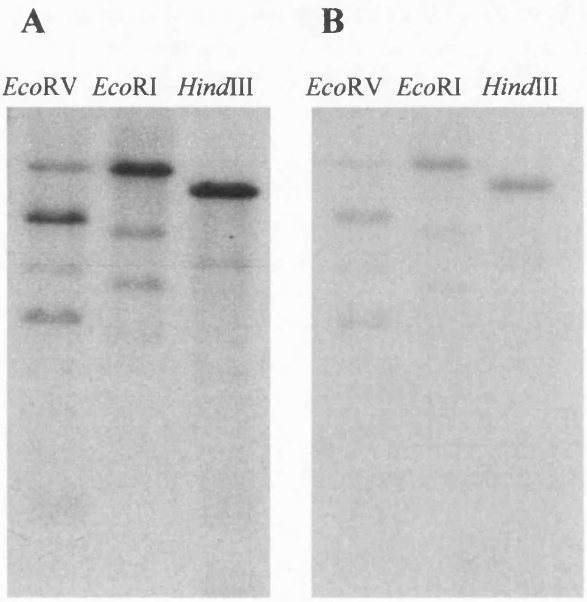


Figure 5.3.1 Southern blot hybridisation with MecCuZnSOD-PCR. 10µg of digested genomic DNA was run on a 0.8% TAE gel overnight and Southern blotted according to standard procedures. Hybridisation was carried out overnight at 55°C. Panel A = Low stringency wash (2X SSC, 0.1% SDS at 55°C for 2x 20 minutes). Panel B = High stringency wash (0.1X SSC, 0.1% SDS at 60°C for 20 minutes).

For the low stringency wash, several hybridising bands of variable intensity were detected, suggesting the occurrence of a small CuZnSOD gene family in cassava. After washing at high stringency, 2 hybridising bands remain in the *Eco*RV lane, whilst a single band remains in the *Eco*RI and *Hind*III lanes, suggesting the gene encoding MecCuZnSOD occurs in the cassava genome as a single copy.

5.4 Detection of superoxide dismutase isoforms by polyacrylamide gel electrophoresis

For the detection of cassava root SOD isoforms, total protein was extracted from air freighted roots of cultivar MPER 337 at 0, 1, 2, 3 and 4 days after injury, as described in section 2.7.15.7 30µg aliquots of each sample were then electrophoresed on an isoelectric focussing (IEF) polyacrylamide gel (Ampholine PAG plate pH 3.5 – 9.5 Amersham Pharmacia). After running, a starch gel overlay method was used according to Manchenco (1994) as described in section 2.7.15.7. The colorimetric substrates used were NBT (nitroblue tetrazolium) and PMS (phenazine methosulphate) allowing the detection of SOD isoforms as clear areas on a blue background. Results are shown below (figure 5.4.1) and indicated the occurrence of 4 SOD isoforms in the cassava root. No significant changes in isoform pattern or intensity was detected during the post harvest period.

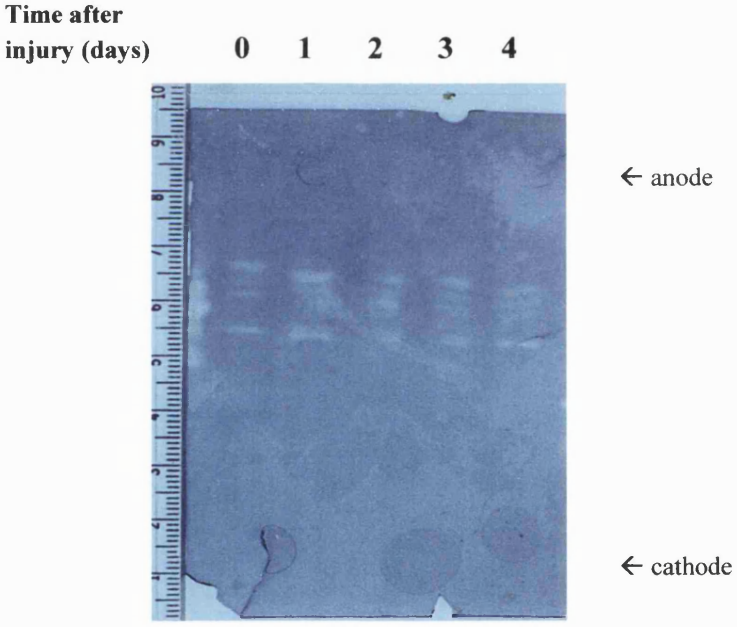


Figure 5.4.1 Detection of SOD isoforms in the cassava storage root. Total protein was extracted from roots of cultivar MPER 337 at 0, 1, 2, 3 and 4 days after injury. Isoform detection was carried out by a starch gel overlay method as described in section 2.7.15.7.

5.5 Tissue localisation of MecCuZnSOD-PCR transcript expression

Transcript accumulation of MecCuZnSOD in roots, petiole and leaves of the cassava plant was examined by northern blotting as described in section 2.7.10. Total RNA was extracted from plants of cultivar MDOM5 at CIAT, Colombia. Root samples were taken

on the day of harvest. Expression was detected in all tissues examined, and was similar to the transcript expression profile of mSOD1 which was likewise detected in all tissues with the exception of adventitious roots of cassava (Lee *et al.* 1999).

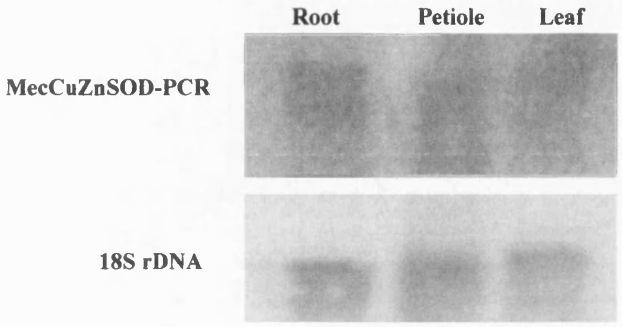


Figure 5.5.1. mRNA expression of MecCuZnSOD-PCR in different tissues of the cassava plant. As a control for equivalent loading the blot was stripped and re-probed with a cDNA encoding cassava 18S rRNA (lower panel).

5.6 Effect of pruning treatment, ethylene and jasmonic acid on superoxide dismutase transcript accumulation

Accumulation of the transcript corresponding to MecCuZnSOD-PCR in response to pre-harvest pruning, treatment with ethephon and methyl jasmonate was examined by northern blotting as described in section 2.7.10. Treatments were carried out as described in section 2.7.10.8. Total RNA was extracted from storage roots of cultivar MCOL 22 at CIAT, Colombia. Root samples were processed or treated immediately after harvest.

For the pre-harvest pruning treatment, roots were obtained from plants which had been pruned by removal of the stem and all leaves approximately 30cm from the ground 2 weeks prior to harvest. Control RNA samples were prepared from similar non-pruned plants harvested at the same time. For the ethylene treatment, root slices were incubated in the ethylene generating compound ethephon (Sigma) (0.02% in sterile water) for 24 hours in the dark. Control slices were incubated in water alone. For the methyl jasmonate treatment, root slices were incubated for 24 hours in the dark in methyl jasmonate (Sigma) (500μM in 0.1% ethanol. Results are shown in figure 5.6.1. The transcript was moderately induced by pre-harvest pruning, but was unaffected by ethylene or methyl jasmonate treatment. This expression profile differs from that of mSOD1, since the mSOD1 transcript was up-regulated by ethephon treatment (Lee *et al.* 1999) and indicates that as in maize (Scandalios 1997), the members of the cassava cytosolic CuZnSOD gene family may contain differing regulatory regions.

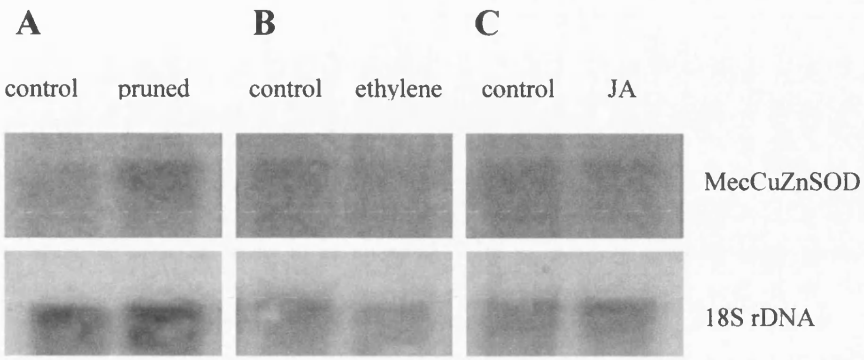


Figure 5.6.1 mRNA expression of cytosolic CuZnSOD MecCuZnSOD-PCR in response to different treatments. For all treatment panels control samples are shown on the left, experimental treatment samples are shown on the right. Panel A = Pre harvest pruning treatment. Panel B = Ethephon treatment. Panel C = Methyl jasmonate treatment.

5.7 Comparative transcript expression in different cultivars

Superoxide dismutase transcript accumulation for the MecCuZnSOD-PCR cytosolic CuZnSOD was examined in a range of cultivars showing differing susceptibility to PPD. Total RNA was extracted from storage roots of cultivars MCOL 22 (highly susceptible), MNGA 2 (intermediate susceptibility), MDOM 5 (low susceptibility) and CMC 21772 (variable susceptibility). Roots were freshly harvested from the field and were then injured by removal of the proximal and distal ends of the roots and cutting of 2 "V" shaped incisions through the epidermis along the length of the root. The root ends were covered with parafilm and roots were stored in an open air shed. Under the experimental conditions used here, visible symptoms of PPD occurred in the storage roots of all cultivars within 24 hours after harvest and the PPD response progressed rapidly. Rates of deterioration in MCOL 22, MNGA 2 and MDOM 5 were roughly similar, whilst CM 21772 showed slightly less pronounced deterioration. Northern blotting and hybridisation experiments were carried out as described in section 2.7.10 using MecCuZnSOD-PCR as a probe. Results are shown in figure 5.7.1.

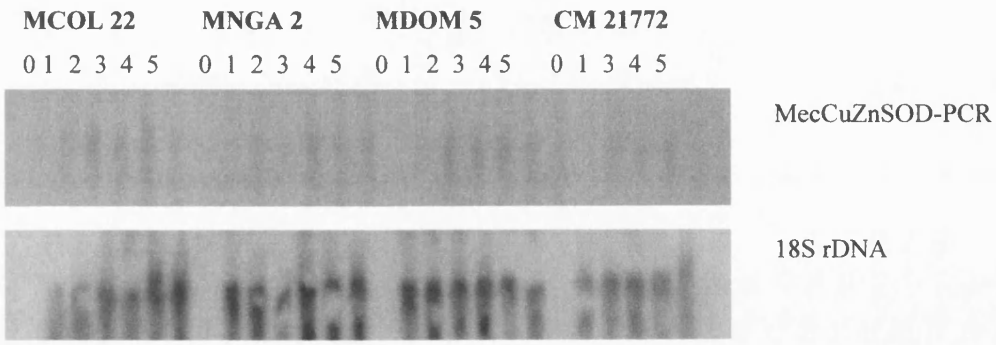


Figure 5.7.1 mRNA transcript accumulation of cassava superoxide dismutase MecCuZnSOD-PCR during the post harvest period. The time after harvest is indicated in days. 10μg aliquots of each sample were run on a denaturing formaldehyde gel and northern blotted according to standard procedures (Sambrook *et al.* 1989). As a control for equal loading the same blot was stripped and re-hybridised with an 18S rDNA probe (lower panel).

5.8 Conclusions and discussion

Results presented here describe the generation of a PCR probe corresponding to a cassava CuZnSOD. The sequence and predicted polypeptide of MecCuZnSOD showed high similarity to the 3' region of other plant cytosolic CuZnSODs (Bowler 1994). At the amino acid level, the sequence presented here showed highest similarity to a papaya (*Carica papaya*) cytosolic CuZnSOD (100% pairwise amino acid identity) over the region compared. Similarity to the mSOD1 deduced polypeptide (Lee *et al.* 1999) was 99% (pairwise amino acid identity) over the region compared. Comparison of MecCuZnSOD-PCR and mSOD1 at the nucleotide level indicates the sequences are not identical and represent different members of a small gene family of CuZnSODs in the cassava genome. In other plant systems (maize and *Arabidopsis*) CuZnSODs may occur as gene families comprising 3 - 5 members (Scandalios 1997, Kliebenstein *et al.* 1998). Polyacrylamide gel electrophoresis indicated the presence of 4 SOD isoforms which are expressed in the cassava storage root. Isoform bands showed no significant changes in intensity or pattern over a 5 day post-harvest period, suggesting that superoxide dismutases in the cassava storage root do not show an altered regulatory pattern during the development of PPD. Similarly, the transcript expression profile of the cognate CuZnSOD to MecCuZnSOD-PCR showed little change in expression profile during post-harvest storage.

The transcript was expressed in all tissues examined, and showed slight up-regulation in response to pre-harvest pruning treatment, but was unaffected by ethylene or methyl jasmonate. Taken together these data would suggest that altered regulation of SOD isoforms, and of the CuZnSOD encoded by MeCuZnSOD-PCR, does not play a significant part during PPD of the cassava storage root.

CHAPTER SIX:
REACTIVE OXYGEN SPECIES AND NON ENZYMATIC ANTIOXIDANTS PRODUCED DURING POST-HARVEST DETERIORATION

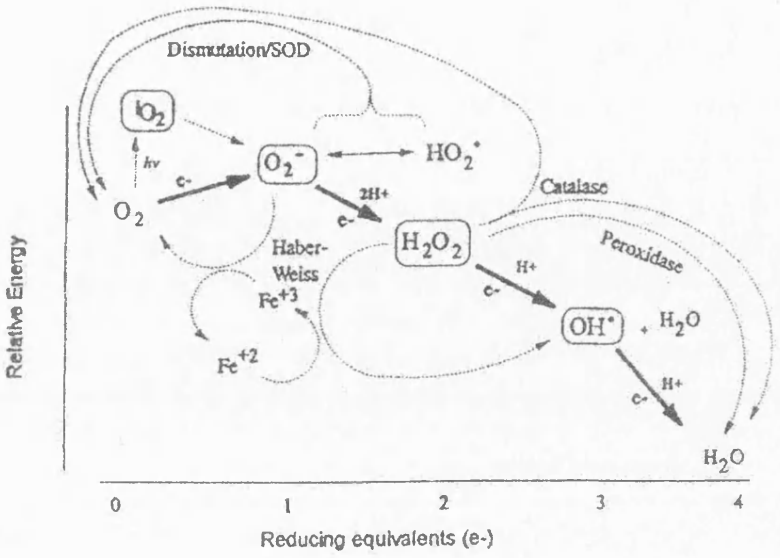
6.1 Introduction and literature review

6.1.1 Types of Reactive Oxygen Species (ROS) found in plants

The terms active or reactive oxygen species (AOS or ROS) or reactive oxygen intermediates (ROI) are used primarily, but not exclusively, to refer to reactive molecules that result from the reduction of molecular di-oxygen (O_2). Whilst molecular di-oxygen is relatively un-reactive and non toxic it becomes reactive once its electron structure is altered (Baker and Orlandi 1995). Although many ROS are oxygen radicals, i.e. contain an unpaired electron, several, such as singlet oxygen ($^1O_2$) and hydrogen peroxide H_2O_2 are not. Many ROS may act as oxidants – interacting with other molecules to gain an electron, however not all oxidants are radicals (e.g. H_2O_2), and not all radicals are strong oxidants (e.g $O_2^{\bullet -}$ is a weak oxidant only).

The main forms of ROS thought to exist in plants and the major inter-conversion pathways between them are summarised in figure 6.1. Singlet oxygen ($^1O_2$) is a highly reactive form of di-oxygen (O_2) in which one of the un-paired electrons of ground state di-oxygen [σ_{1s} ($\downarrow\uparrow$) σ^*_{1s} ($\downarrow\uparrow$),σ_{2s} ($\downarrow\uparrow$) σ^*_{2s} ($\downarrow\uparrow$), π_{2p} ($\downarrow\uparrow$) ($\downarrow\uparrow$),σ_{2p} ($\downarrow\uparrow$) π_{2p} ($\downarrow$) ($\downarrow$), σ^*_{2p} ( )] is promoted to an orbital of higher energy with or without an inversion of spin. It is not therefore a free radical but it is highly reactive since it contains a vacant molecular orbital. The superoxide radical ($O_2^{\bullet -}$) results from a 1 electron reduction of molecular di-oxygen and is reactive due to the presence of its single unpaired electron, particularly in hydrophobic environments such as the interior of membrane bilayers (Thompson *et al.* 1987). The hydroperoxyl radical ($HO_2^{\bullet}$) is the conjugate acid of the superoxide radical. It is formed under acidic conditions ($O_2^{\bullet -} + H^+ \rightarrow HO_2^{\bullet}$) and is more lipophilic and more reactive than the superoxide radical, however it is believed that at physiological pH only a small proportion of superoxide would occur in this form. Hydrogen peroxide (H_2O_2) is a relatively stable oxidant, but unlike other ROS is long lived and is capable of crossing lipid bilayers (Thompson *et al.* 1987, Baker and Orlandi 1995). The hydroxy radical ($OH^{\bullet}$) is formed via reduction of hydrogen peroxide and is one of the most reactive ROS although it consequently has only a short half life (Bowler *et al.* 1994, D'Auzac 1996).

In addition, other secondary radicals such as the alkoxy radical ($RO^{\bullet}$), peroxy radical ($ROO^{\bullet}$) and organic hydroperoxides (ROOH) may be formed *in planta* via lipid peroxidation initiated either enzymatically (e.g. lipoxygenase) or by ROS such as $O_2^{\bullet -}$ or $OH^{\bullet}$ (Thompson *et al.* 1987, Larson 1995, Chapple 1997).



1) Superoxide/hydroperoxyl equilibrium:

$O_2^{•-} + H^+ \leftrightarrow HO_2^•$ (pKa=4.8)

2) Dismutation:

$HO_2^• + O_2^{•-} + H^+ \rightarrow H_2O_2 + O_2$ ($k_2 \sim 8 \times 10^5\ M^{-1}\ s^{-1}$)

$O_2^{•-} + O_2^{•-} + 2H^+ \rightarrow H_2O_2 + O_2$ ($k_2 \sim 0.2\ M^{-1}\ s^{-1}$)

3) Haber-Weiss Reaction:

$Fe^{3+} + O_2^{•-} \rightarrow Fe^{2+} + O_2$

$Fe^{3+} + O_2^{•-} \rightarrow Fe^{2+} + O_2$

$O_2^{•-} + H_2O_2 \rightarrow OH^• + OH^- + O_2$

4) Catalase:

$H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2$

5) Peroxidase:

peroxidase + H_2O_2 → compound 1 + H_2O

compound 1 + RH_2 → compound 11 + $RH^•$

compound 11 + RH_2 → peroxidase + $RH^•$ + H_2O

Figure 6.1 Reactive oxygen species derived from molecular di-oxygen and likely inter-conversion pathways thought to exist in plant systems (after Baker and Orlandi 1995).

6.1.2 Cellular targets, effects and roles of ROS in plant systems

Although molecular oxygen is itself relatively unreactive, its ease of conversion to more reactive, partially reduced forms, necessitates a battery of cellular defences - ranging from specific enzymes such as catalase, peroxidase and superoxide dismutase to non enzymatic antioxidants and quenchers – in order to maintain cellular homeostasis.

In plant systems, the major initial sources of ROS during normal metabolism are the production of superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) via electron "leakage" from electron transport chains (e.g. photosystems I and II, the mitochondrial electron transport chain, electron transport chains located in the ER, membrane, peroxisomes and nuclear envelope); and production of singlet oxygen $^1O_2$ during photosynthesis by transfer of excitation energy from triplet chlorophyll to molecular di-oxygen. (reviewed in Bartosz 1997). Although neither $O_2^{\bullet -}$ nor H_2O_2 is highly reactive at physiological concentrations, toxicity *in vivo* arises from their role as substrates in the iron catalysed Haber-Weiss reaction (see equation 3 figure 6.1) (Bowler *et al.* 1994). The hydroxy radical ($OH^{\bullet}$) formed via this reaction and its derivatives are amongst the most reactive species known, and react indiscriminately with cellular macromolecules causing lipid peroxidation, protein denaturation and DNA damage.

Under stress conditions, increased ROS formation often occurs through perturbation of such metabolic paths, and cellular damage arising from environmental stresses are often caused by oxygen radicals. Both plant and animal systems are susceptible to cell death and damage resulting from oxidative injury. Examples of this type include xenobiotics and herbicides such as paraquat which are subject to redox cycling, abiotic stresses such as drought or high light intensity, auto-oxidising chemicals such as SO_2, ozone and UV radiation (Foyer *et al.* 1997, Bartosz 1997).

A large body of recent research has focussed on the controlled production of ROS, particularly $O_2^{\bullet -}$ and H_2O_2, in processes such as senescence, wounding and pathogen defence – in particular the HR (hypersensitive response) and SAR (systemic acquired resistance).[1]

The pivotal role of ROS in plant defence against pathogens first came to light in 1983 when Doke and colleagues detected an "oxidative burst" in higher plants analogous to the respiratory burst of mammalian neutrophils (Doke 1983a, b). The oxidative burst is defined as a rapid production of ROS - in particular $O_2^{\bullet -}$ and H_2O_2 -in response to external stimuli (Wojtaszek 1997). Working on potato tuber tissues and incompatible races of *Phytophthora infestans,* Doke and colleagues showed that the HR (hypersensitive response) was accompanied by high levels of $O_2^{\bullet -}$ production which began within minutes of fungal penetration. Sustained $O_2^{\bullet -}$ generation was not observed in compatible (i.e. disease causing) interactions. Subsequent research has shown the

[1] The HR refers to the rapid, localised programmed host cell death which occurs at the site of attempted infection by incompatible pathogens. The resultant necrotic lesions impede pathogen invasion and allow resistance to occur. SAR refers to the process whereby localised infection with certain necrotising pathogens i.e. those which induce a HR, provides enhanced resistance of the entire plant to secondary infection by a range of pathogens.

oxidative burst is a characteristic feature of the plant pathogen response. The initial ROS formed is $O_2^{\bullet -}$ which is rapidly converted to H_2O_2. The process is initiated rapidly after pathogen recognition, and involves a series of signal transduction events that typically include fluxes in Ca^{2+} and other ions, phospholipases, GTP binding proteins, protein kinases and phosphatases (Doke 1993b, Dixon *et al.* 1994, Miura *et al.* 1995, Scheel 1998, Desikan *et al.* 1996, 1999). Studies with cell suspension cultures indicate that in compatible reactions leading to disease, a short monophasic oxidative burst is detected, whilst in incompatible reactions leading to a HR a biphasic, sustained production of ROS is observed (Baker and Orlandi 1995, Low and Merida 1996, Doke *et al.* 1996, Mehdy *et al.* 1996, Allan and Fluhr 1997, Van Camp *et al.* 1998). In the case of compatible reactions, the sustained production of ROS is inhibited by suppressor glucans of the pathogen (Doke *et al.* 1996). The timing of ROS production detected varies depending on the experimental system used. In suspension cultured cells the initial (phase I) production of ROS usually begins within 1-2 minutes of elicitation, reaches a maxima several minutes later and declines by 30-60 minutes after elicitation. The second (phase II) peak which occurs for incompatible reactions only, begins within 1-1.5 hours and declines by around 6 hours after elicitation (Wojtaszek 1997). When plant segments are used to study the oxidative burst, O_2^- generation is commonly reported to occur within minutes and decline after around 10 hours. H_2O_2 production is evident within 2-4 hours, with peaks reported at 6-12 hours, or as late as 14-17 hours (Wojtaszek 1997, Vallelian-Bindschedler 1998). Although much of the research on the oxidative burst has been carried out using fungal pathogens and elicitors, an oxidative burst during defence responses to bacterial, viral and nematode pathogens or elicitors, as well as host cell wall derived elicitors such as oligogalacturonide (OGA) has been described (reviewed in Wojtaszek 1997, D'Auzac 1996, Doke *et al.* 1997, Legendre 1993). In addition, several studies have indicated an oxidative burst in response to wounding (Olsen and Varner 1993, Schopfer 1994, Wanatabe and Sakai 1998, Orozco-Cardenas and Ryan 1999); mechanical stress (Cazale *et al.* 1998, Legendre 1993, Collen and Pederson 1994, Yahraus *et al.* 1995); and heat or cold shock (Vallelian-Bindschedler 1998) suggesting that the oxidative burst may form part of a general stress defence pathway (Scott *et al.* 1999).

According to presently proposed models, the ROS generated during the oxidative burst play several complex and overlapping roles in facilitating plant defence. Essentially these may be summarised as i) cell wall strengthening, ii) induction of defence related genes, and iii) triggering of host cell death.

Considerable evidence has accumulated that the H_2O_2 generated by the oxidative burst functions in the oxidative cross linking of cell wall proteins, particularly hydroxyproline rich glycoproteins (HRGPs), via the formation of isodityrosine cross links catalysed by peroxidase. The process results in the insolubisation of these proteins within the cell wall and occurs rapidly, often insolubisation is complete within 20-30 minutes of elicitation (Mehdy *et al.* 1996). Cell wall HRGPs may act as nucleation sites for subsequent deposition of lignin and other phenolics, again lignin polymerisation involves H_2O_2 and peroxidase mediated crosslinking (Adam 1995, Milosovic and Slusarenko 1996). Such cell wall strengthening is believed to act as a physical barrier to impede pathogen ingress.

During the HR, up-regulation of genes related to defence such as HRGPs (physical defence), PR proteins such as glucanase and chitinase (pathogen defence), and PAL (multi-component defence allowing accumulation of phytoalexins, SA and lignin precursors) is commonly reported. In addition up-regulation of genes related to ROS scavenging is commonly described in tissues surrounding necrotic lesions. H_2O_2 has been proposed to function as a second messenger resulting in the activation of such genes. The precise mechanism is unclear, but may involve lipid peroxidation products and/or direct or indirect activation of transcription factors analogous to mammalian NFκB. Changes in cell membrane permeability and cell membrane degradation are commonly observed in cells undergoing HR (D'Auzac 1996, Adam 1995), and may be mediated either directly by $O_2^{\bullet -}$ or indirectly by the iron catalysed Haber-Weiss formation of $OH^{\bullet}$ from H_2O_2 and $O_2^{\bullet -}$ (figure 6.1 equation 3) (Thompson *et al.* 1987). Alternatively, mechanisms involving lipoxygenase catalysed membrane degradation, utilising H_2O_2 from the oxidative burst could be envisioned. Sequences closely approaching the 13bp binding site for the mammalian H_2O_2 activated transcription factor NFκB have been identified in the *Arabidopsis* PAL1 and GST6 promotors, both of which are induced by H_2O_2 in *Arabidopsis* (Desikan *et al.* 1998). In mammalian systems, NFκB occurs in the cytoplasm in a non DNA binding form, associated with an inhibitory subunit IκB. H_2O_2 causes dissociation of IκB, resulting in activation and nuclear translocation of NFκB via an indirect mechanism involving a metabolite of H_2O_2 or an intracellular reaction induced by H_2O_2 (Schreck *et al.* 1991). Diverse signals, often associated with pathological conditions, are able to induce gene expression via H_2O_2 activation of NFκB and it is likely that similar mechanisms may exist in plants, allowing the plant to activate defensive and/or PCD (programmed cell death) genes downstream of the oxidative burst. The production of ethylene is a common response of

plants to wounding and pathogenesis and has been described in cassava after a lag of around 6 hours after injury (Plumbley *et al.* 1981). Recent results (Wanatabe and Sakai 1998), have indicated the wound induced up-regulation of an ACC synthase transcript in winter squash (*Cucurbita maxima*) tissue blocks that could be inhibited by DPI (an inhibitor of superoxide generating NADPH oxidases); and potentiated by superoxide generating systems. Thus gene modulation via the plant stress hormone ethylene could be mediated via an initial oxidative burst.

Host cell death may be triggered by direct toxic effects of H_2O_2 or its metabolites such as $OH^{\bullet}$. Lipid peroxidation and consequent membrane breakdown is thought to contribute to this process. In addition, recent evidence suggests that cell death during the HR may represent a form of programmed cell death, rather than simply necrotic cell death triggered by oxidative insult. For example, in soybean (*G.max*) suspension cells H_2O_2 induced cell death that showed features characteristic of apoptosis including formation of DNA "ladders", and characteristic cell morphology (Levine *et al.* 1996). In *Arabidopsis*, H_2O_2 and elicitor induced cell death could be inhibited by cycloheximide and cordycepin – inhibitors of protein and RNA synthesis respectively- indicating that cell death was an active process (Desikan *et al.* 1998). A number of studies have indicated that the production of ROS was necessary but not sufficient to induce host cell death (Glazener *et al.* 1996, Jabs *et al.* 1997, Van Camp *et al.* 1998, Dorey *et al.* 1999). Recently, salicylic acid (SA) and nitric oxide (NO) have been proposed to act synergistically with ROS to promote cell death (Van Camp *et al.* 1998, Dangl 1998, McDowell and Dangl 2000). The mechanism by which SA stimulates cell death is unclear, however, since SA can bind and inactivate catalase (Chen *et al.* 1993) it may function via signal amplification processes, although this has not been conclusively demonstrated. An alternate mechanism via SA potentiation of a regulatory phosphorylation step during the oxidative burst has been proposed (Alvarez 1997). NO can also drive PAL gene expression, thereby stimulating SA biosynthesis (Dangl 1998, McDowell and Dangl 2000).

With regard to the wound and mechanical stress induced oxidative burst in particular, non chemical signal perception via a putative mechanosensor has been proposed (Low and Schroeder 1996). More recently, Orozco-Cardenas and Ryan (1999) have reported the transient wound induced production of hydrogen peroxide within 1 hour after injury in leaves of 14 higher plant species. Hydrogen peroxide was detected primarily in the vascular tissues and maximised between 4-6 hours after injury before declining. The production of hydrogen peroxide was inhibited by DPI, implicating the participation of

an NADPH oxidase in the formation of ROS. In a series of experiments to dissect the signalling pathway leading to this oxidative burst, their research has implicated systemin as the primary wound signal. Methyl jasmonate and plant cell wall derived oligogalacturonic acid (OGA) fragments produced by endogenous, wound inducible polygalacturonidase were also required for the production of hydrogen peroxide but act downstream of systemin.

6.1.3 Sources, production and location of superoxide and hydrogen peroxide in plant systems

At least 2 major models have been proposed for the *in planta* generation of superoxide ($O_2^{\bullet -}$), in particular regarding the source of $O_2^{\bullet -}$ production during the oxidative burst. Based on work on the oxidative burst generated during incompatible host-parasite interactions between potato (*Solanum tuberosum*) and potato blight (*Phytophthora infestans*), Doke and colleagues proposed a plasma membrane NADPH oxidase analogous to that of mammalian leucocytes (Doke *et al.* 1985, 1996). The enzyme catalyses the transfer of electrons from the internal side of the plasma membrane to molecular oxygen on the external side of the membrane resulting in formation of $O_2^{\bullet -}$. Bolwell *et al.* (1995) present an alternate model for the oxidative burst in which superoxide is produced by a cell wall bound peroxidase. Although peroxidases are generally considered as scavengers rather than producers of ROS, the ability of plant peroxidases to produce hydrogen peroxide (H_2O_2) has been known since the 1980s. Mader and Amberg-Fisher (1982) demonstrated the production of H_2O_2 by a cell wall bound peroxidase in a reaction that consumed NADH and was stimulated by Mn^{2+} and certain phenols. Essentially, the peroxidase utilises H_2O_2 to oxidise NADH to the free radical $NAD^{\bullet}$, which in turn reduces O_2 to $O_2^{\bullet -}$. Subsequent reduction of $O_2^{\bullet -}$ to H_2O_2 may occur spontaneously or catalysed by the enzyme superoxide dismutase as in figure 6.1. (Thompson *et al.* 1987). The reaction is strongly pH dependant showing maximum production at neutral or basic pH depending on the peroxidase isoforms being studied (Bolwell *et al.* 1999). Inhibitor studies to date have been somewhat inconclusive in deciding between the 2 models. Several studies indicate that the oxidative burst can be inhibited by DPI (diphenyliodonium), an inhibitor of flavin oxidases including the mammalian NADPH oxidase (Doke 1985, Auh and Murphy 1995, Desikan *et al.* 1996). Soybean and *Arabidopsis* proteins immunologically related to the mammalian NADPH complex have been reported (Desikan *et al.* 1996, Tenhaken *et al.* 1995) and cDNAs encoding plant homologues of mammalian NADPH oxidase

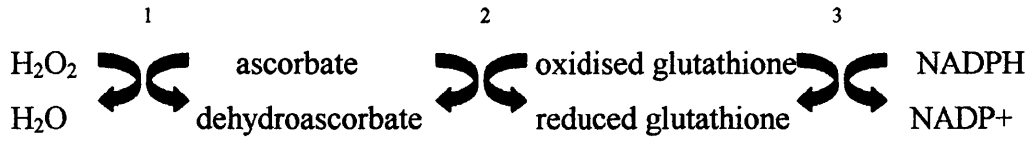
components have been isolated (Groom *et al.* 1996). Other studies however provide support for the cell wall peroxidase model. For example, in studies on elicitor treated soybean suspension cells a partial inhibition with DPI, but a complete inhibition with KCN – an inhibitor of peroxidases – was observed (Mithofer *et al.* 1997). Similarly, in elicitor treated french bean cells an oxidative burst which was accompanied by consumption of NADH but not NADPH and was inhibited by KCN has been described (Bolwell *et al.* 1995, 1999, Bolwell 1996). A requirement for the presence of a cell wall localised component has been demonstrated in carrot, since elicitor treated protoplasts were unable to produce an oxidative burst (Bach *et al.* 1993). Given these ambiguous results several authors have suggested that more than 1 mechanism may operate in tandem to produce the oxidative burst, a suggestion which has been supported by experimental evidence. For example, in studies using confocal imaging with the fluorescent probe dichlorofluorescein to study generation of ROS in elicited tobacco cells, (Allan and Fluhr 1997) the fungal elicitor cryptogein induced ROS production from a plasma membrane flavin containing oxidase (since production was inhibited by DPI) suggesting an NADPH oxidase or xanthine oxidase. Amine and polyamine compounds produced during pathogenesis and wounding induced a DPI insensitive oxidative burst at the cell periphery, a location and inhibitor profile consistent with a cell wall bound peroxidase or amine oxidase type of enzyme.

Several other oxidoreductases are also known to catalyse the reduction of O_2 to $O_2^{\bullet -}$. These include xanthine oxidase, NADPH-cytochrome P450 reductase and mitochondrial NADH dehydrogenase (Thompson *et al.* 1987). In addition, an alternate route for superoxide production has been proposed via lipoxygenase catalysed degradation of membrane lipids (Adam *et al.* 1995, Croft *et al.* 1990). However, this mechanism has generally been dismissed as too slow to be relevant to initial signalling (Alvarez and Lamb 1997).

6.1.4 Secondary metabolites produced in the cassava storage root after harvest and their potential function as antioxidants

In addition to enzymatic ROS scavengers, plants employ a variety of low molecular weight antioxidants in protection against potentially damaging ROS. These include hydrophilic compounds present in the aqueous phase and hydrophobic compounds located in cell membranes. Hydrophobic compounds include flavanoids; coumarins; cinnamic acids such as ferulic acid; polyphenols such as lignins and tannins; glutathione

and ascorbate. Ascorbate and glutathione participate in the Halliwell-Asada cycle as below:



Reactions 1, 2 and 3 are catalysed by ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase respectively and the cycle is primarily responsible for removal of ROS in the chloroplast stroma (Bartosz 1997, Thompson *et al.* 1987). Hydrophobic compounds which occur in cell membranes include tocopherols such as vitamin E; carotenoids such as β-carotene, xanthophylls, flavonoids and vitamin D. (Gille and Sigler 1995, Larson 1995, Bartosz 1997, Dornberg and Davies 1999).

The major route for non photochemical oxidative damage in plant cells is the interaction of molecular oxygen with free radicals, resulting in free radical chain reactions such as lipid peroxidation. Many plant secondary metabolites, especially the electron rich phenolic compounds are capable of acting as antioxidants. They may function as primary or chain breaking antioxidants, which react with lipid radicals converting them to more stable products; and secondary or preventative antioxidants which reduce the rate of chain initiation or decompose hydroperoxides to non radical species (Larson 1987, Amiot *et al.* 1997). Essentially, such plant phenolics may act on several levels. For example they may act as free radical terminators or reducing agents; they may act to out-compete radicals by virtue of being easily oxidised compounds; or they may chelate metal ions, thereby preventing formation of the hydroxyl radical ($OH^{\bullet}$) via the Haber-Weiss reaction. In addition some phenolics, particularly flavenoids such as epicatechin gallate and epigallocatechin, are capable of inhibiting lipoxygenase activity (Amiot *et al.* 1997).

A number of phenolic compounds which could potentially act as antioxidants have been shown to accumulate in the cassava storage root after harvest. These include the fluorescent coumarin compounds scopoletin, scopolin and esculetin as well as 2 unidentified scopoletin and esculetin conjugates; and the flavonoids (+)catechin and gallocatechin (Rickard and Gahan 1983, Tanaka *et al.* 1983, Uritani *et al.* 1984). Other phenolic compounds which have been identified in the cassava storage root, although they are do not accumulate to high levels in response to injury include the coumarin esculetin, and the flavonoids catechin gallate and rutin (Buschmann *et al.* 2000a, b).

Lignins and condensed tannins have also been described (Rickard *et al.* 1979, Rickard and Gahan 1983).

Although oxidation of phenolic compounds by free radicals serves to minimise lipid peroxidation, oxidation of phenolic compounds via enzymatic (peroxidase and polyphenol oxidase) and non enzymatic reactions (Cheigh *et al.* 1995) can result in the formation of brown or black pigmentation of plant tissue, for example during the HR (Goodman *et al.* 1986) and during post harvest storage (Amiot *et al.* 1997). The catechins and their initial reaction products are effective antioxidants and effective browning substrates (Cheigh *et al.* 1995), whilst the coumarin scopoletin can be oxidised by peroxidase giving an insoluble blue reaction product (Edwards *et al.* 1997).

In other plant systems, phenolic secondary metabolites have been proposed to play a variety of roles including functions as antimicrobial phytoalexins, as antioxidants, as defensive compounds against insect herbivory, as well as roles as plant cell wall components. The coumarin scopoletin is known to accumulate in solonaceous plants in response to wounding and infection, and it has been identified as an antimicrobial compound in several plant systems including rubber (*Hevea brasiliensis*) (Giesmann *et al.* 1986) and in cassava (Rodriguez *et al.* 2000). Other studies, suggest that it functions as an antioxidant during plant defence responses (Chong *et al.* 1999). The flavon-3-ol epichatechin has been proposed to act as an antioxidant in avocado (Ardi *et al.* 1998), whilst epigallocatechin gallate has been characterised as showing antioxidant and antifungal roles (Rice-Evans 1995, Li *et al.* 1999).

6.1.5 Chapter summary

The detection of a wound induced oxidative burst which occurs rapidly in cassava storage roots after injury is described. Transient production of superoxide (O_2^-) was detected histochemically by an NBT (nitro blue tetrazolium) reduction method, and sites of production were examined using light microscopy. The generation of hydrogen peroxide (H_2O_2) was detected using the DAB (3,3 diaminobenzidine tetrahydrochloride) reagent, and localisation was examined by light microscopy. Levels of hydrogen peroxide produced in the cassava storage root after injury were quantified in two cultivars showing contrasting susceptibility to PPD using a chemiluminescence method. Thin Layer Chromatography techniques were used to examine secondary metabolite profiles in the cassava storage root after harvest and to determine their potential functioning as antioxidants.

6.2 Detection and localisation of superoxide in cassava root tissue after harvest

For the detection of superoxide (O_2^-) in wounded cassava storage roots a modification of the method of May *et al.* (1996) was used as described in section 2.7.11.1. Roots were harvested and immediately cut into transverse slices of ~2mm thickness. At each time point the tissue section was vacuum infiltrated with 0.05% NBT (nitroblue tetrazolium) in 10mM K_2HPO_4 buffer (pH 6) and incubated for 15 minutes. Superoxide production was indicated by the formation of a blue precipitate.

Initial experiments were carried out using duplicate storage roots of cultivar MCOL22 grown under greenhouse conditions at Bath University. Results are shown below (figure 6.2.1). Superoxide production, as indicated by the formation of a blue precipitate, occurred within 15 minutes after wounding and had declined to undetectable levels by 6-7 hours after wounding. Diffuse purple/blue staining was observed in the root parenchyma, with a band of darker blue staining commonly observed surrounding in the cambium.

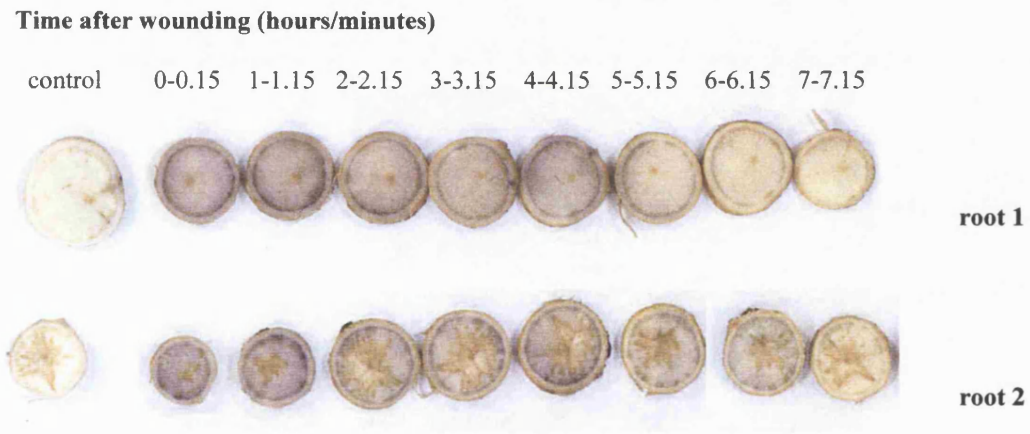


Figure 6.2.1 *In situ* detection of O_2^- production in wounded cassava storage roots (cultivar MCOL22). Tissue slices at each time point were vacuum infiltrated with 0.05% NBT in 10mM K_2HPO_4 buffer (pH 6) and incubated at room temperature for 15 minutes in the light. Control reactions for each time point were infiltrated with buffer alone and never showed blue coloration. An example of a control reaction is shown on the right.

For experiments carried out at CIAT, Colombia, superoxide production was examined in a range of cultivars showing differing susceptibility to PPD. For each cultivar, 3 different roots were freshly harvested, immediately cut into sections and vacuum infiltrated as previously described. Results are shown in figure 6.2.2. Superoxide formation was again detected within 15 minutes after wounding but was sustained over a slightly longer period than had been observed under Bath conditions, with some root

samples showing detectable superoxide production at 10 hours after injury. Similar macroscopic localisation was observed with intense staining commonly observed in the cambium. Considerable variation in staining intensity was observed both between different roots of the same cultivar, and between different cultivars.

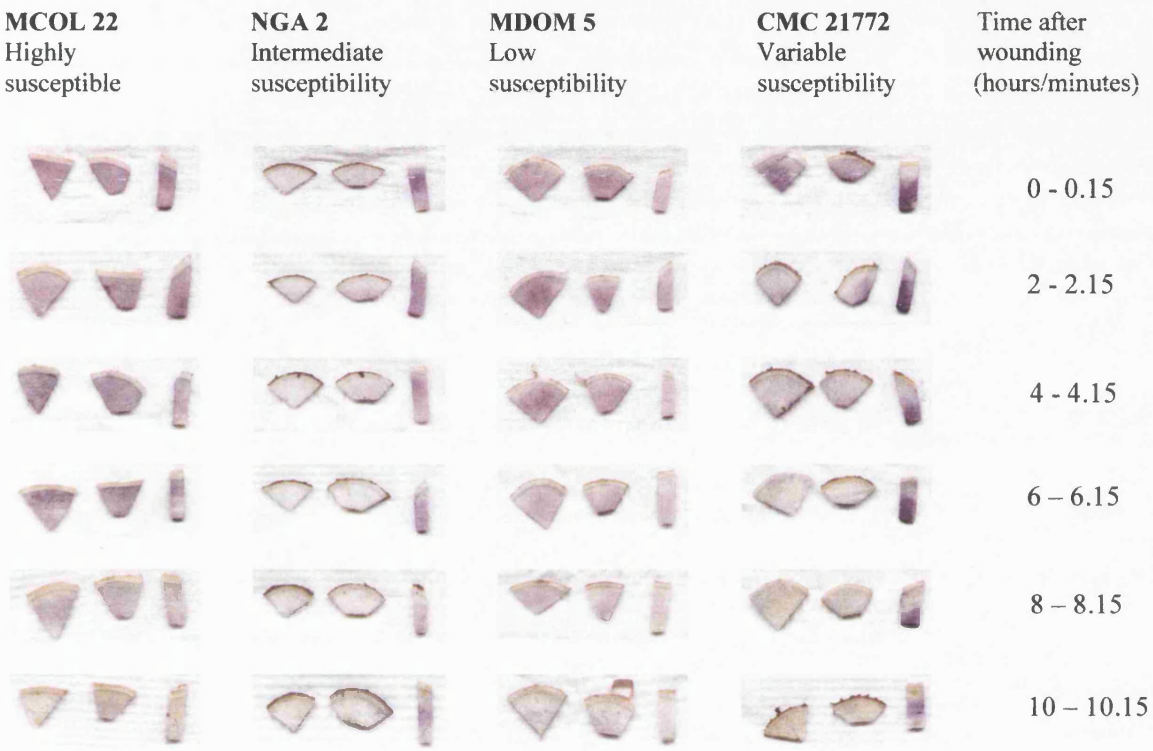


Figure 6.2.2 *In situ* detection of O_2^- production in wounded cassava storage roots at CIAT, Colombia. For each cultivar 3 different roots were examined for superoxide production over a time course of 10 hours according May *et al.* (1996). For each panel ▽ = root 1 ▽ = root 2 ▯ = root 3.

Since experiments carried out both at Bath and at CIAT had indicated macroscopic localisation of superoxide production to particular tissue types within the storage roots, localisation at the cellular level was examined by light microscopy according to the method of Ros Barcelo *et al.* (1998) as described in section 2.7.11.2. The method is essentially similar to that of May *et al.* (1996), however the sections are not vacuum infiltrated and are incubated in a staining solution at higher pH.

Results are shown in figure 6.2.3. Within the root storage parenchyma, pale blue staining was primarily visible at cell walls and cell wall junctions. Strong cell wall staining was not observed in the cortical parenchyma. Occasional cells showing intense blue staining throughout the cell were observed in the storage parenchyma and in

packaging parenchyma surrounding the xylem vessels (see panel A figure 6.2.3). Closer examination of the cambium area of tissue sections showed intense blue staining associated with individual cells within the cambium (see panels B and C figure 6.2.3). These strongly staining cells were primarily although not exclusively located at the exterior part of the cambium just underlying the cortex.

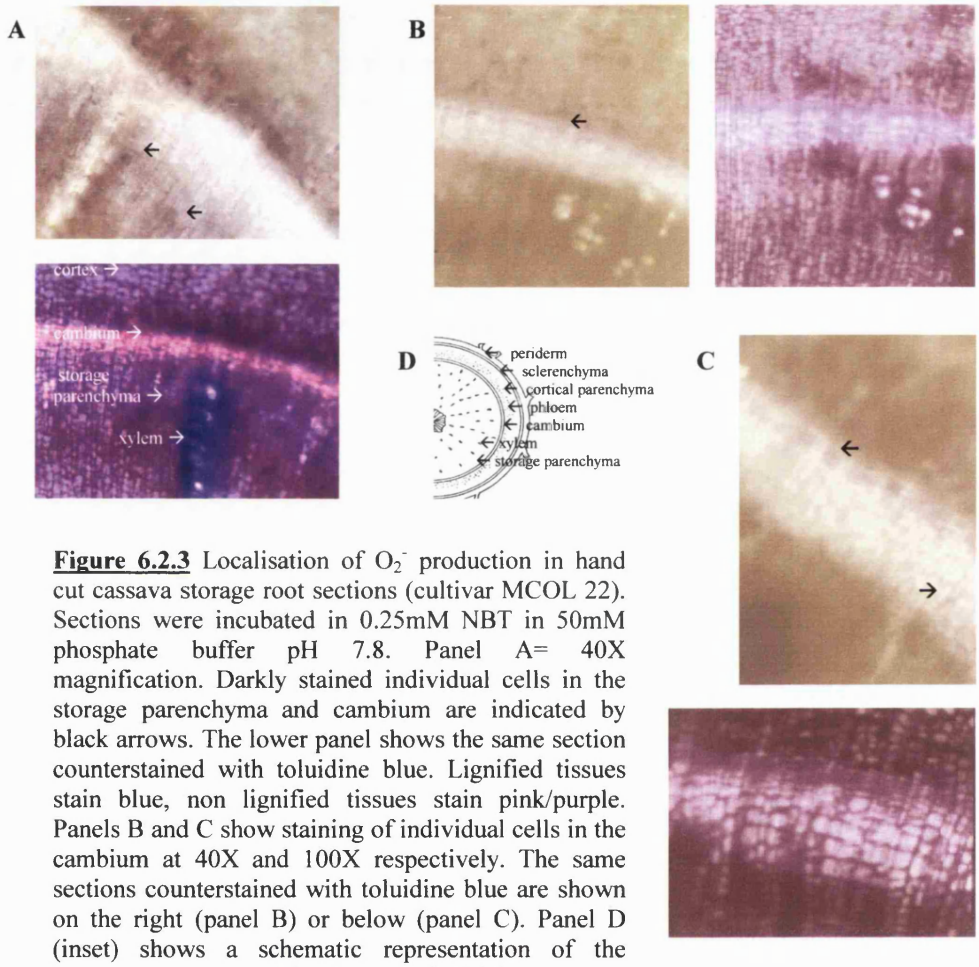


Figure 6.2.3 Localisation of O_2^- production in hand cut cassava storage root sections (cultivar MCOL 22). Sections were incubated in 0.25mM NBT in 50mM phosphate buffer pH 7.8. Panel A= 40X magnification. Darkly stained individual cells in the storage parenchyma and cambium are indicated by black arrows. The lower panel shows the same section counterstained with toluidine blue. Lignified tissues stain blue, non lignified tissues stain pink/purple. Panels B and C show staining of individual cells in the cambium at 40X and 100X respectively. The same sections counterstained with toluidine blue are shown on the right (panel B) or below (panel C). Panel D (inset) shows a schematic representation of the cassava storage root (after Hunt *et al.* 1977).

6.3 Detection, localisation and quantification of hydrogen peroxide in wounded cassava roots after harvest

For the *in situ* detection of hydrogen peroxide in cassava storage roots the method of Thordal-Christensen *et al.* (1997) was used as described in section 2.7.11.3. At each time point, transverse root slices of width ~ 2mm were vacuum infiltrated with 2mg/ml DAB (3,3 diaminobenzidine tetrahydrochloride) and incubated at room temperature for 3 hours before documentation. Production of hydrogen peroxide was indicated by the

formation of a brown precipitate. For control reactions similar tissue slices at each time point were vacuum infiltrated with DAB (2mg/ml) and 10mM ascorbate (scavenger of hydrogen peroxide).

Initial experiments were carried out in Bath using greenhouse grown roots of cultivar MCOL 22 over a time course of 36 hours. Immediately after harvest the proximal and distal ends of the root were removed and covered with a small piece of parafilm and the root was injured by cutting a small "V" shaped incision through the epidermis and cortex along the length of the root with a fresh razor blade. Production of hydrogen peroxide was evident within 3 hours after wounding and was still detected, although at lower levels, at 33-36 hours after wounding. Staining was initially primarily localised to the cortical parenchyma and internal storage parenchyma just underlying the cambium, with little staining observed in the central part of the root (see figure 6.3.1 0-3 to 12-15 hours). Both the timing and location of hydrogen peroxide accumulation suggests that it results via conversion from superoxide. The timecourse of hydrogen peroxide production was again similar to that described in barley leaves in response to heat treatment (Vallelian- Bindshedler *et al.* 1998).

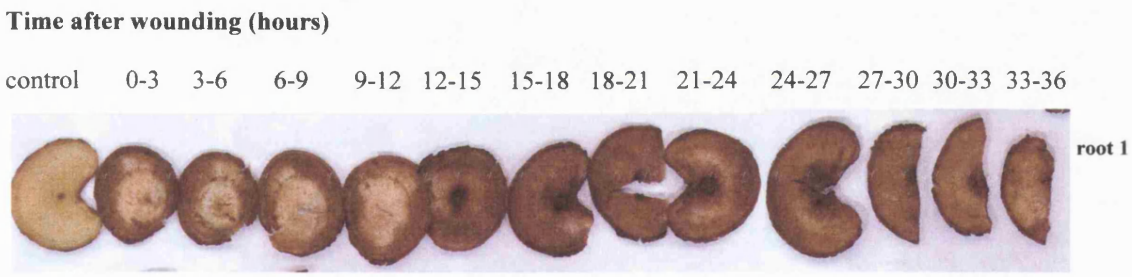


Figure 6.3.1 *In situ* detection of H_2O_2 production in wounded cassava storage root (cultivar MCOL22). Tissue slices at each time point were vacuum infiltrated with 2mg/ml DAB and incubated at room temperature for 3 hours. Control reactions for each time point were infiltrated with DAB (2mg/ml) and 10mM ascorbate. An example of a control reaction is shown on the left.

For subsequent experiments at Bath, time course experiments were carried out over a period of days rather than hours using duplicate roots of cultivars showing differing susceptibility to PPD. Vacuum infiltration and control reactions were carried out as before and results are shown below (figure 6.3.2). Production of hydrogen peroxide was again evident within 3 hours after injury, increased at 1 day (24-27 hours) and had declined to low levels by 2 days (48-51 hours) after injury. Staining was again initially most intense in the cortex and storage parenchyma underlying the cortex. For these

experiments no apparent difference was observed between roots showing high susceptibility (MCOL 22) and low susceptibility (MDOM 5) to PPD.

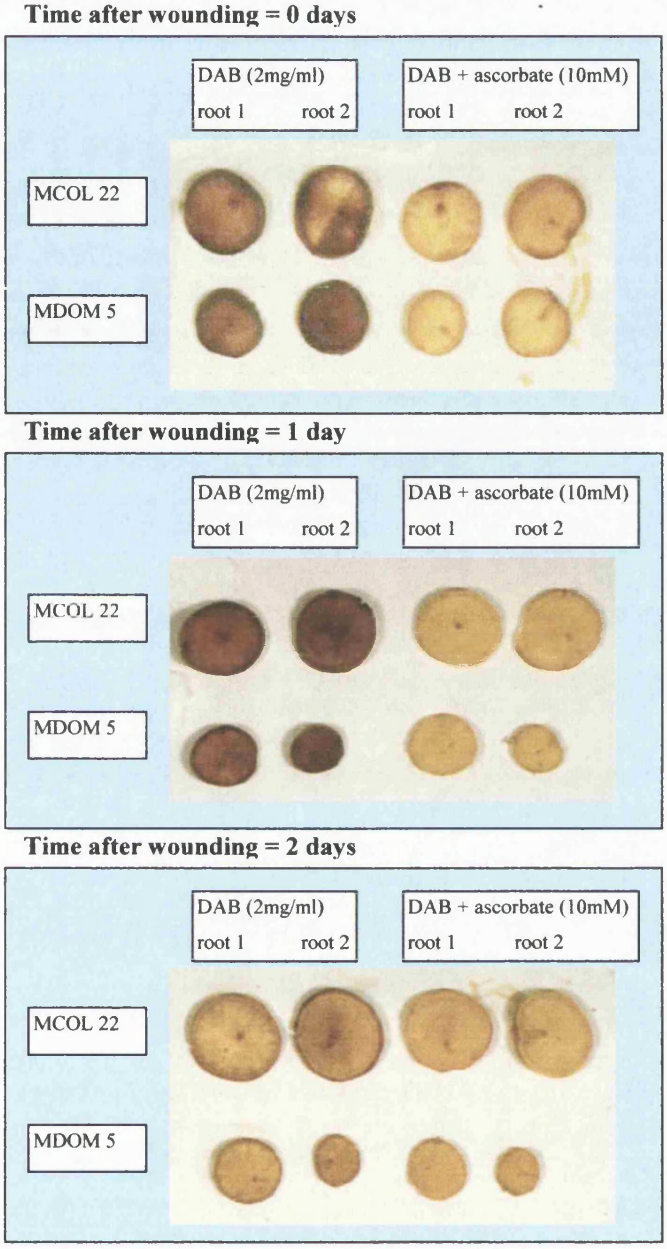


Figure 6.3.2 *In situ* detection of H_2O_2 production in wounded cassava storage roots showing differing susceptibility to PPD. Cultiver MCOL 22 was used for high PPD response and MDOM 5 for low PPD response. For each pane,l experimental reactions are shown on the left, control reactions are shown on the right.

The DAB reagent gives intense staining at hydrogen peroxide levels of 1-10μM, (Thordal-Christensen *et al.* 1997), suggesting that relatively high levels of hydrogen peroxide are produced in the cassava root after wounding. In order to allow quantification of levels of hydrogen peroxide produced in the cassava storage root after

injury, a modification of the method of Warm and Laties (1982) was used as described in section 2.7.12. For this experiment, 3 roots for each of 2 cultivars were obtained from greenhouse grown plants at the University of Bath. MCOL 22 shows high susceptibility to PPD whilst MVEN 77 has been characterised as showing low PPD susceptibility. Results are shown below (figure 6.3.3). Roots of both cultivars showed a peak in accumulation of hydrogen peroxide within 24 hours after injury followed by a steady decline. Again, no significant difference was observed between roots showing high susceptibility (MCOL 22) and low susceptibility (MVEN 77) to PPD. Levels of hydrogen peroxide were comparable to those detected by Cazale *et al.* (1998) in tobacco cell suspension cultures in response to mechanical and hypoosmotic stress.

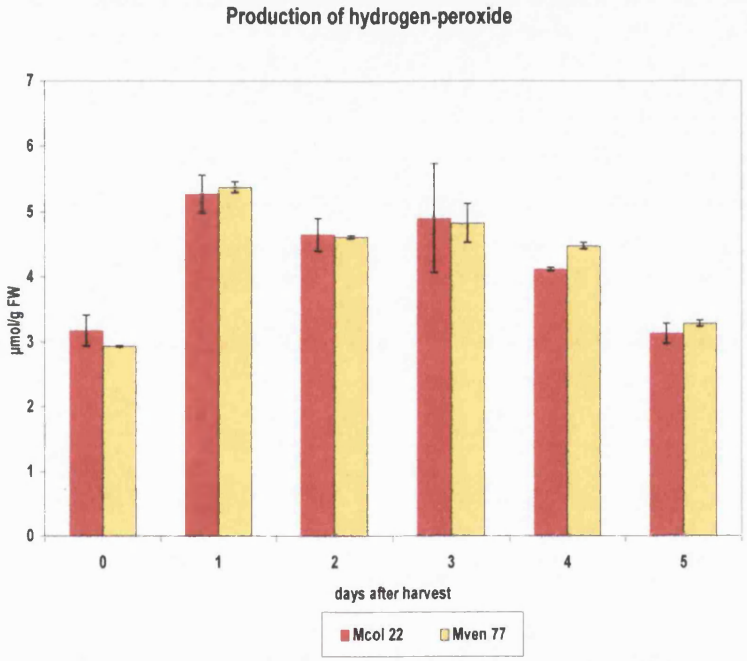


Figure 6.3.3 *In vitro* quantification of H_2O_2 production in wounded cassava storage roots showing differing susceptibility to PPD over a storage period of 5 days. Cultivar MCOL 22 was used for high PPD response and MVEN 77 for low PPD response. Each column represents the mean of 3 roots per cultivar (mean ± SD).

When the DAB method described in section 2.7.11.3 was used to examine hydrogen peroxide accumulation at the cellular level using light microscopy, similar tissue localisation was observed (figure 6.3.4). Within 3 hours after harvesting and injury, intense staining was observed particularly in the cortical parenchyma and storage parenchyma underlying the cortex. Staining was especially strongly associated with xylem vessels and surrounding parenchyma (panel A). At higher magnification (panels C and D) staining was predominantly observed at the cell walls, cell wall junctions and middle lamellae. These data were in agreement with earlier microscopic observations (Buschmann pers com.) using the starch-KI method of Olsen and Varner (1993),

however the starch-KI method was not used here as it did not give sufficiently intense staining to allow adequate documentation (Buschmann *et al.* 2000).

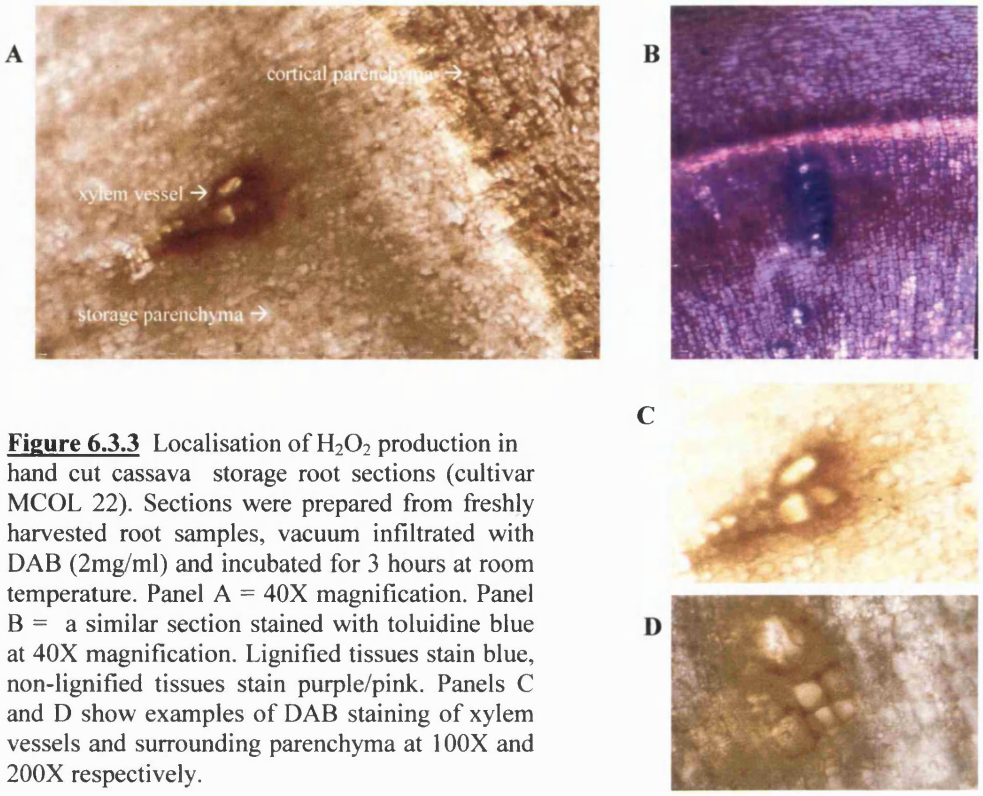


Figure 6.3.3 Localisation of H_2O_2 production in hand cut cassava storage root sections (cultivar MCOL 22). Sections were prepared from freshly harvested root samples, vacuum infiltrated with DAB (2mg/ml) and incubated for 3 hours at room temperature. Panel A = 40X magnification. Panel B = a similar section stained with toluidine blue at 40X magnification. Lignified tissues stain blue, non-lignified tissues stain purple/pink. Panels C and D show examples of DAB staining of xylem vessels and surrounding parenchyma at 100X and 200X respectively.

For experiments carried out at CIAT, Colombia, triplicate root samples were obtained from plants of 4 cultivars showing varying susceptibility to PPD. Roots were injured by removal of the proximal and distal ends, and cutting of 2 "V" shaped incisions along the length of the root through the periderm and cambium. The ends of the roots were covered with parafilm and samples were stored in an open air shed. Under these storage conditions the relative rate of PPD progression was MCOL 22 ≅ MNGA 2 > MDOM 5 > CM21772. At each time point, hand cut root slices were vacuum infiltrated with DAB (2mg/ml), or with DAB and 10mM ascorbate for the control reactions. All samples were incubated for 3 hours before documentation. Results are shown below (figure 6.3.4) and were slightly ambiguous as compared to previous experiments carried out in Bath, particularly for the low susceptibility cultivars MDOM 5 and CMC 21772. For all cultivars, only slight accumulation of H_2O_2 was observed within the first 3 hours after harvest. All root samples showed an increase at 1 day after injury followed by a pronounced decline at day 2, as had been expected, in cultivars MCOL 22, MNGA 2 and to a lesser extent in MDOM 5. However, although at least some of the brown coloration in the later time points after harvest could be attributed to PPD related

browning of the roots, cultivars MDOM 5 and CM 21772 showed apparent sustained production of H_2O_2 relative to controls at days 3 and 5 after injury. These results cannot be easily reconciled with experiments at Bath which had indicated no significant differences between high and low PPD response cultivars using both qualitative (DAB infiltration) and quantitative (chemiluminescence) methods. Two possible hypothesis might be suggested. Firstly, the experiment was discontinued on day 6 due to visible secondary microbial deterioration, it is possible that the sustained production of H_2O_2 could an early response to pathogen attack. Secondly, since the PPD response progresses more quickly under Colombian storage conditions than Bath storage conditions, the experiments carried out in CIAT, Colombia, might show a second peak of H_2O_2 accumulation that is not observed at Bath over the same time frame. Commonly, roots stored under Bath conditions show symptoms of vascular streaking at 2 to 4 days after injury depending on cultivar. Under Colombian storage conditions, all roots studied showed vascular streaking within 24 hours after harvest and the response progressed rapidly. Since roots do produce a wound periderm under certain conditions, although far more slowly than other root crops (Ravi 1996, Pounti-Kerlas 1998) it is conceivable that the H_2O_2 detected in the later stages could represent second peak of H_2O_2 produced by the root in wound sealing lignification type reactions. Although there is no evidence in the literature, one might suspect that the wound healing and sealing capabilities of the less susceptible cultivars might be more pronounced.

Detection of Hydrogen Peroxide

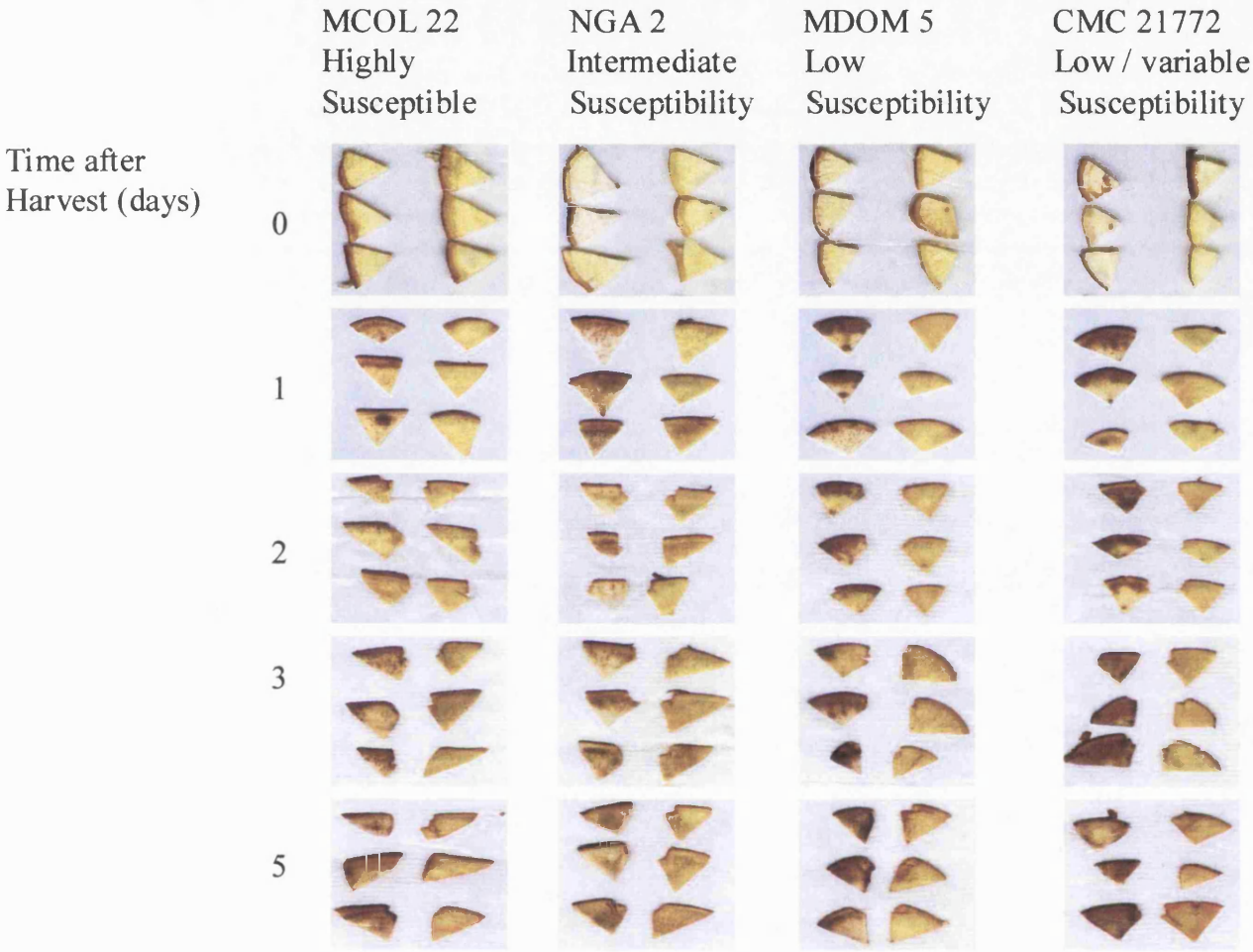


Figure 6.3.4 *In situ* detection of H_2O_2 production in cassava storage roots of 4 cultivars showing differing susceptibility to PPD. For each panel, triplicate experimental reactions (DAB 2mg/ml) are shown on the left, triplicate control reactions (DAB 2mg/ml + 10mM ascorbate) are shown on the right.

6.4 Non enzymatic antioxidants produced in the cassava root during the post harvest period

Ethanolic root extracts were prepared from storage roots of cassava (cultivars MCOL 22 and MNGA 2) over the post harvest period as described in section 2.7.13.1. Component compounds were separated by high performance thin layer chromatography on HPTLC silica gel F_{254} plates as described in section 2.7.13.2. The solvent system used was chloroform: ethyl acetate: methanol (2:2:1). After documentation of the plates under UV light (245nm and 366nm), the plate was sprayed with a fine mist of DPPH (1,1 diphenyl-2-picryl-hydrazl) in order to allow detection of antioxidant bands according to Takao *et al.* (1994). The DPPH reagent is a free radical with a strong pink/purple colour that fades on interaction with free radical scavengers. Thus, antioxidant compounds with free radical scavenging capability can be identified as clear areas on a pink/purple background.

For the experiment shown in figure 6.4.1 100µl aliquots of ethanolic root extracts prepared from cultivar MNGA 2 at 0, 1, 2, 4, 6 and 8 days after injury were separated by TLC and antioxidants detected with the DPPH reagent. When viewed under UV light, the coumarin compounds scopoletin and scopolin were readily identified due to their characteristic fluorescence under UV (figure 6.4.1 panels A and B). When the plate was sprayed with DPPH reagent, five bands with antioxidant properties were identified by the formation of clearance zones (figure 6.4.1 panel C). When separated by TLC, each compound has a characteristic R_f (retention factor) value calculated as the distance travelled by the band / the distance travelled by the solvent front. The R_f value is constant for a particular compound with a particular solvent system, and thus allows comparison between different experiments. The calculated R_f values here were band a = R_f 0.68, b = R_f 0.56, c = R_f 0.34, d = R_f 0.23 and e = R_f 0.04. The R_f values for the fluorescent bands assumed to be scopoletin and scopolin were R_f 0.56 and R_f 0.17 respectively, and were in agreement with known R_f values for these compounds with this solvent system (Buschmann pers. com.). Thus the weak antioxidant band b (R_f 0.56) could be identified as scopoletin.

For subsequent experiments, ethanolic root extracts prepared from roots of cassava cultivars showing contrasting susceptibility to PPD were separated by TLC. Cultivar MCOL 22 shows high susceptibility to PPD whilst MNGA has been characterised as showing intermediate susceptibility. Aliquots of the coumarin compounds scopolin, scopoletin and esculin; and the flavin-3-ols (+)catechin and gallocatechin, which are known to accumulate in the cassava storage root after harvest

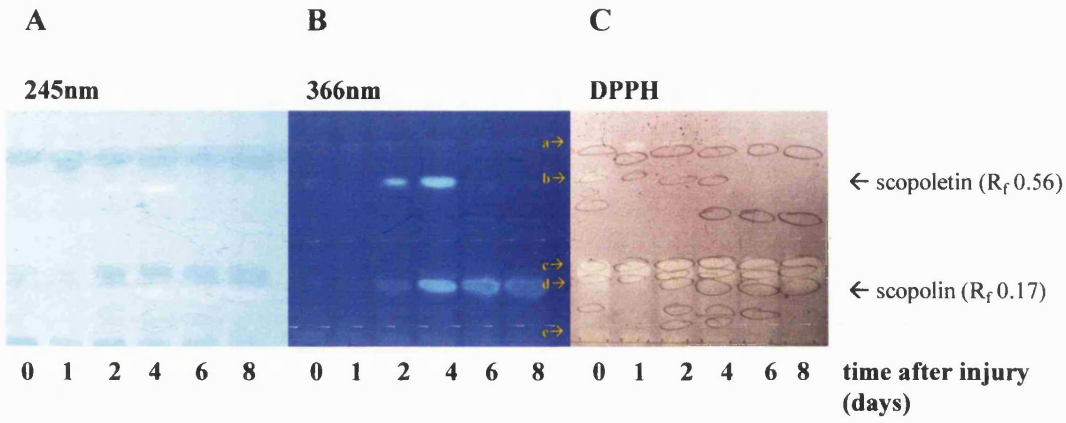


Figure 6.4.1 TLC separation and detection of antioxidant compounds in ethanolic root extracts of cultivar MNGA 2 during post harvest storage. Panel A = TLC plate viewed under UV light (254nm), Panel B = TLC plate viewed under UV light (366nm), Panel C = TLC plate sprayed with DPPH. The time after injury of the root is shown below the baseline. Bands showing free radical scavenging activity are indicated by yellow arrows.

were run alongside the experimental samples as references. Results are shown in fig 6.4.2. Since the documentation of the previous experiment, shown in figure 6.4.1, had been disappointing (as several bands visible when the TLC plate was observed under UV light could not be clearly seen in the photographs), attempts were made to optimise documentation. Therefore, the amount of experimental samples used was increased from 100µl to 150µl, and photographs were taken using Fugicolor Crystal Archive Paper film rather than tungsten balanced Kodak Ectachrome 160T 35mm slide film. As may be seen from figure 6.4.2, documentation under UV light was far superior. Spraying with DPPH indicated that of the reference compounds (+)gallocatechin, (+)catechin, scopoletin and esculetin are capable of acting as free radical scavengers, although scopoletin was weakly active only. Scopolin showed no free radical scavenging ability. As may be seen in figure 6.4.2, DPPH resolution of antioxidant bands in the experimental samples, particularly bands c and d was less pronounced than previously, and may be related to oxidation of the root extracts during storage. Of the previously identified bands, band a (R_f 0.69) remains unidentified since it did not correspond to any standard. However, the yellowish coloration of this band when viewed under UV light at 366nm (panel B, figure 6.4.2) could indicate that it may be a

carotenoid or other highly coloured compound. Band b (R_f 0.56) was confirmed as corresponding to the coumarin scopoletin. Band c (R_f 0.34) did not correspond to any standards run on this plate. However, it may tentatively be identified as epicatechin gallate, since it had a similar expression profile and R_f value to a previous standard run using the same solvent system (Rodriguez pers. com). Band d (R_f 0.23) was identified as the 3-flavonol gallocatechin. Band e (R_f 0.04), although again not corresponding to a standard run on this plate, could be identified as the flavon-3-glycoside rutin, since it had an identical R_f value to a previous standard separated using this solvent system (Rodriguez pers. com).

Overall, no marked differences were observed between the 2 cultivars, although extracts from the high PPD response cultivar MCOL 22 appeared to have slightly higher antioxidant capacity as indicated by more pronounced clearance on this side of the TLC plate. When reference and experimental lanes are compared, results indicate that the flavonoid (+) gallocatechin, and the coumarin scopoletin are capable of acting as antioxidant free radical scavengers and may fulfil this role *in vivo*. These data would be consistent with previous research indicating that scopoletin functions as an antioxidant *in planta* (Chong *et al.* 1999); and studies in cassava indicating accumulation of scopoletin and gallocatechin to relatively high levels during PPD (Tanaka *et al.* 1983, Buschmann *et al.* 2000). In contrast, the coumarin scopolin, although it showed high levels of accumulation did not show free radical scavenging ability in experimental samples or in reference lanes. The flavonol (+) catechin and the coumarin esculetin, although capable of acting as strong antioxidants as indicated by the strong clearance of the reference samples, did not show prominent antioxidant properties in the experimental samples. Again, these data are consistent with previous studies indicating that these secondary metabolites accumulate at relatively low levels (Tanaka *et al.* 1983, Uritani *et al.* 1984, Buschmann *et al.* 2000), and with the observation in this study that UV fluorescent bands corresponding to esculetin were not seen. Two additional bands, identified as the flavonoids rutin and epicatechin gallate showed antioxidant capability in the experimental samples, and have been characterised as antioxidants in the literature (Larson 1987, Amiot *et al.* 1997).

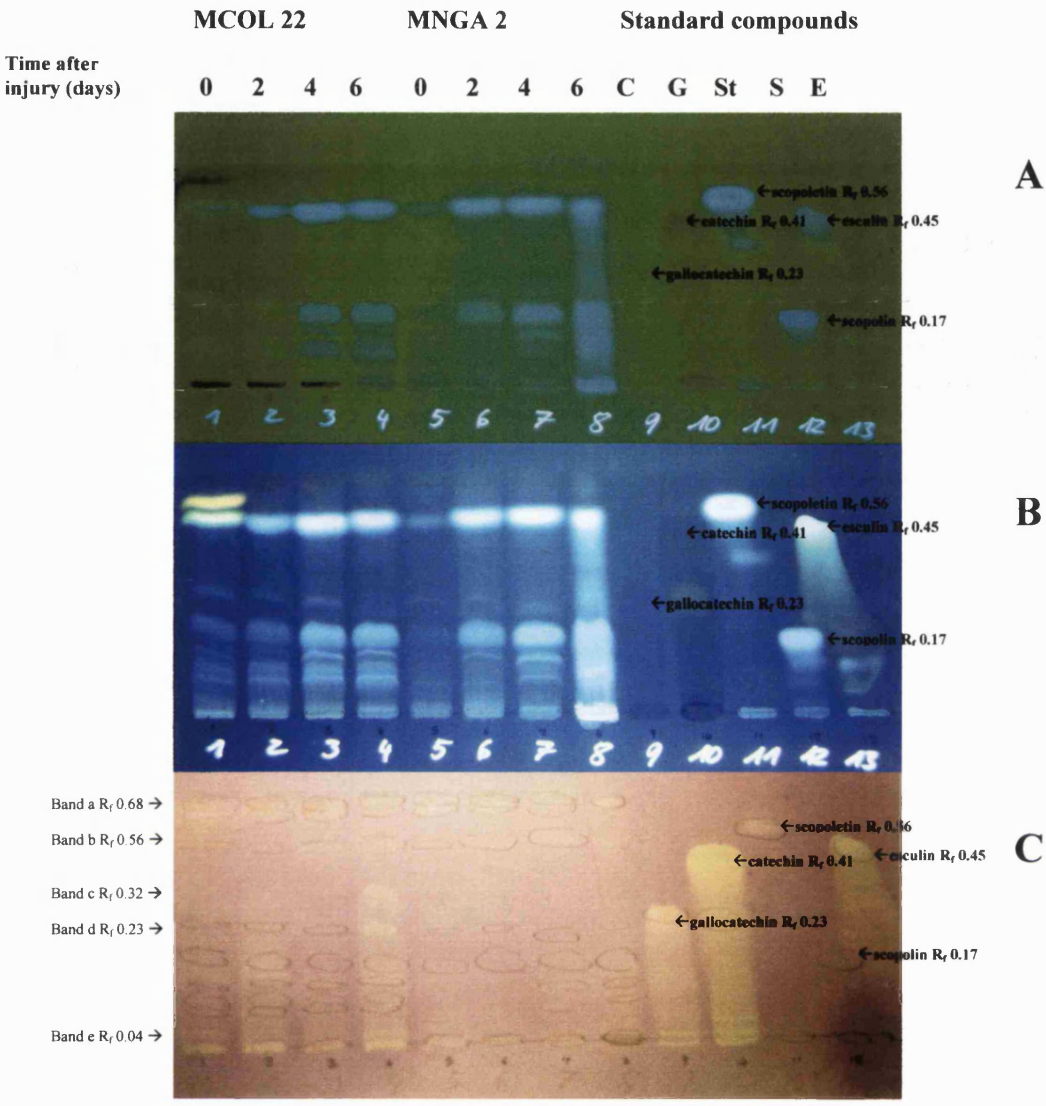


Figure 6.4.2 TLC separation and detection of antioxidant compounds in ethanolic root extracts of cultivar MCOL 22 (high susceptibility to PPD) and MNGA 2 (intermediate susceptibility) during post harvest storage. Panel A = TLC plate viewed under UV light (254nm), Panel B = TLC plate viewed under UV light (366nm), Panel C = TLC plate sprayed with DPPH. The cultivar, root time after injury and standard lanes are shown at the top of the figure. G = gallocatechin, C = (+) catechin, St = scopoletin, S = scopolin, E = esculetin. Bands showing free radical scavenging activity are indicated on the left of panel C.

6.5 Conclusions and discussion

Results presented here indicate the occurrence of a transient, wound induced oxidative burst in cassava storage roots. Superoxide (O_2^-) was produced within 15 minutes after injury and had declined to low levels by 6-10 hours after injury. Hydrogen peroxide (H_2O_2) was detected in roots within 3 hours after injury, and showed a peak of accumulation within 24-27 hours after injury before declining. The timing of this transient production of ROS was similar to that reported for a heat induced oxidative burst in barley (*H.vulgare*) leaves (Vallelian-Bindschedler 1998).

At the macroscopic level, superoxide (O_2^-) production was observed throughout the root parenchyma, with a darkly staining band commonly observed in the region of the cambium. Little staining was observed in the cortex. When examined by light microscopy, staining was associated with cell walls throughout the parenchyma and a ring of darkly stained individual cells was observed in the exterior part of the cambium. In the cassava storage root this part of the cambium gives rise to the secondary phloem (Cabral *et al.* 2000). Given a recent proposed model for wound induced ROS production via a systemin mediated pathway (Orozco-Cardenas and Ryan 1999) this location is interesting, since systemin is phloem mobile (Schaller and Ryan 1995). Occasional single darkly staining cells were observed within the root storage parenchyma and packaging parenchyma surrounding the xylem vessels. Again this location is interesting since "microbursts" of AOS production, occurring in the foci of a single to a few cells and commonly adjacent to vascular bundles, have been described in distal leaves of *Arabidopsis* during SAR (Van Camp *et al.* 1998, Alvarez *et al.* 1998). Such microbursts were often followed by cell death events with morphological features of programmed cell death.

At the macroscopic level H_2O_2 accumulation occurred initially in the cortex, cambium and region of storage parenchyma just underlying the cambium; and spread to internal tissues as the time course progressed. This location would be consistent with the formation of H_2O_2 via dismutation from O_2^- catalysed by an extracellular superoxide dismutase (Scheel 1998). In addition, the part of the storage parenchyma showing earliest accumulation of H_2O_2 corresponds well with the "B" part of the parenchyma showing rapid (within 24 hours) accumulation of high levels of the fluorescent coumarin compounds scopoletin, scopolin and esculetin (Tanake *et al.* 1983, Uritani *et al.* 1983, Uritani *et al* 1984). This "B" part of the parenchyma is the region of the cassava storage root that shows initial formation of symptoms of vascular streaking followed by subsequent tissue browning and cell death (for example see figure 1.4.1) At

the light microscopy level, pronounced staining with the DAB reagent was observed associated with the cell walls and apoplast and was especially strongly associated with the xylem vessels and surrounding xylem parenchyma. Such localisation of H_2O_2 produced during the oxidative burst to cell walls, apoplast and particularly to vascular tissues has been reported in other plant systems (Thordal-Christensen *et al.* 1997, Van Camp *et al.* 1998, Orozco-Cardenas and Ryan 1999). At the microscopic level, strong blue fluorescence due to the coumarin compounds scopolin, scopoletin and esculin has also been reported to be localised to the xylem vessels and spreading into the apoplast of the storage parenchyma (Buschmann *et al.* 2000c).

Quantification of hydrogen peroxide over a time course of 5 days confirmed that a peak of H_2O_2 accumulation occurred within 24 hours after injury in cassava storage roots. Peak levels attained ($\cong$ 5μmol/g FW), were comparable with reported levels for a mechanically induced oxidative burst in other plant systems (Cazale *et al.* 1998). No significant differences in H_2O_2 accumulation were observed between cultivars showing differing susceptibility to PPD. However, qualitative (DAB vacuum infiltration) experiments carried out at CIAT, Colombia indicate that either a second peak or sustained H_2O_2 production of could occur in some cultivars. Further experiments involving quantitative detection of H_2O_2 under Colombia storage conditions or a more prolonged time course carried out under Bath storage conditions would be of interest in order to resolve this matter.

Since H_2O_2 has been demonstrated here to accumulate rapidly in cassava roots after injury and has been implicated as a modulator of defence related gene expression, it would also be of interest to study the effect of H_2O_2 treatment on transcript accumulation of clones isolated from the cDNA library.

Using TLC techniques 5 secondary compounds with potential *in vivo* free radical scavenging properties were detected. Three of these could be identified with a high degree of confidence as the coumarin scopoletin (R_f 0.56), and the flavonoids gallocatechin (R_f 0.23) and rutin (R_f 0.04). A fourth was tentatively identified as the flavonoid epicatechin gallate (R_f 0.34). These data indicate that the cassava storage root contains a range of easily oxidised compounds that could participate as reducing agents (i.e electron donors) in enzymatic or non enzymatic oxidation reactions during the post harvest period.

CHAPTER SEVEN:
CASSAVA cDNA CLONES ASSOCIATED WITH TRANSCRIPTION, TRANSLATION AND PROTEIN TURNOVER

7.1 Introduction and literature review

During the course of this project a number of non-target cDNA clones were obtained, in particular during heterologous screening for peroxidase cDNAs. These include a number of cDNAs encoding enzymes involved in protein turnover – an aspartic protease, a cysteine protease inhibitor, a leucine aminopeptidase of the serine protease family, - and several clones associated with gene transcription and translation – an RNA polymerase subunit, and a translation initiation factor.

Since a component of this project was the generation of cDNA probes for inclusion on the cassava genetic map (Cortez *et al.* 2000) these were subcloned, sequenced and transferred to CIAT. The proteases and protease inhibitor, although unsolicited, were of particular interest since proteases are widely proposed to play a role in senescence processes. Although this avenue has been little explored with regard to cassava PPD, some authors (Passan and Noon 1977, Lalaguna and Agudo 1989) have proposed that the post harvest deterioration of the cassava storage root should be considered as a senescence process.

7.1.1 Senescence and cassava post harvest physiological deterioration

Programmed cell death (PCD) is a broad term used to define processes in which the cell actively promotes its own death via activation of self destructive systems (Gan and Amasino 1997). In contrast, necrosis may be defined as cell death resulting from severe stress or injury – the key distinction being that active gene dependant participation of the cell is not required (Wang *et al.* 1996, Gilchrist 1998). Whilst PCD (termed apoptosis) has been extensively studied in animal systems, programmed cell death in plants has not been extensively studied at either the biochemical or molecular level (Gilchrist 1998, Song and Stellar 2000). It is now generally accepted that many plant developmental processes and stress responses are achieved through PCD, including senescence, xylanogenesis, embryogenesis, abscission zone formation and hypersensitive response (HR) in response to pathogenesis (Gan and Amasino 1997, Greenberg 1996, Song and Stellar 2000). Characteristic features of PCD/apoptosis in animal cells including cytoplasmic shrinkage, nuclear condensation, alterations in membrane structure (membrane blebbing), activation of specific proteases and ordered DNA fragmentation have also been described for plant PCD, although not all in the same plant system (Wang *et al.* 1997, Solomon *et al.* 1999). However no studies of this nature have been carried out in cassava.

Lalaguna and Agudo (1989) described changes in membrane structure and lipid composition during cassava PPD which were characteristic of senescence in other plant systems, and proposed that PPD should be considered as a wound-induced senescence process. In a more recent study using the cDNA-AFLP technique, 70 transcript derived fragments were selected for sequencing based on their expression profile during PPD. Of these, 6% were characterised as PCD related genes (Huang *et al.* 2000). Indeed many of the characteristic features described for plant senescence – inhibition by cycloheximide, selective loss of proteins, membrane changes and increased membrane permeability, increased ethylene production, increased peroxidase activity, increased production of reactive oxygen species, and increased proteolytic activity (Thompson *et al.* 1987, del Rio *et al.* 1998, Panavas and Rubenstein 1998) have been described during cassava PPD in the literature (for review see chapter 1) or have been suggested by this study. However, many of these processes can also be considered as components of plant wound responses and no conclusions should be drawn without further experimentation.

7.1.2 Aspartic proteases

Aspartic proteases (E.C 3.4.23.-) are a widely distributed class of protease present in animals, viruses, microbes, fungi and plants. In mammals, aspartic proteases are localised to the acidic lysosomes (e.g. cathepsin D) or are secreted (e.g. pepsin, renin). In plants they are primarily localised to protein bodies in the vacuole or are secreted to the extracellular space (Rawlings and Bartlett 1995, Mutlu and Gal 1999). They are characterised by specific inhibition by pepstatin A (a hexapeptide from *Streptomyces*); have an acidic pH optimum and preferentially cleave peptide bonds between hydrophobic residues (Faro *et al.* 1999). All known aspartic proteases contain 2 aspartic acid residues at the active site. In most, the catalytic Asp residues are contained within a conserved Asp-Thr -Gly (D-T-G) motif, however the plant aspartic proteases described to date contain Asp-Ser-Gly (D-S-G) at one of the sites. The overall sequence of plant aspartic proteases is similar to their mammalian and microbial counterparts with the exception of a plant specific sequence (PSS) of around 100 residues, found in most plant enzymes with the notable exception of barley nucellin. The function of the PSS is unknown, however it has been proposed to be involved in processing or targeting of plant aspartic proteases (Mutlu and Gal 1999) or to promote association of plant aspartic proteases with cell membranes (Faro *et al.* 1999). The plant aspartic proteases purified to date, like their mammalian counterparts, may be monomeric (e.g. rennin, pepsin) or

dimeric (e.g. cathepsin D). Where the cognate genes or cDNAs have been isolated, the sequences predict that the active heterodimeric enzymes are derived from processing of a single pre-proprotein. Differently sized heterodimeric forms have also been described, for example the *Hordeum vulgare* seed aspartic protease phytepsin comprises 2 isoforms of the enzyme which are derived from the same transcript – a 48 kDa isoform composed of 32 and 16 kDa subunits, and a 40 kDa isoform composed of 29 and 11 kDa subunits. A schematic representation of the *H.vulgare* protease and its cognate peptides illustrating the modular structure of plant aspartic proteases is shown in figure 7.1.1

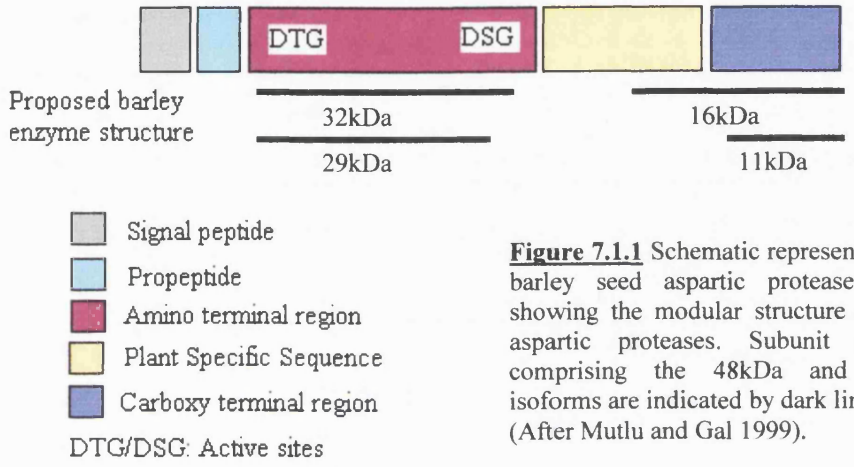


Figure 7.1.1 Schematic representation of barley seed aspartic protease cDNA showing the modular structure of plant aspartic proteases. Subunit peptides comprising the 48kDa and 40kDa isoforms are indicated by dark lines. (After Mutlu and Gal 1999).

All non viral aspartic proteases are synthesised as inactive precursors (zymogens) in which the N-terminal propeptide is bound to the active site cleft (Tang and Wong 1987, Kissil and Kimchi 1998, Kervinnen *et al.* 1999). Subsequent cleavage of the N terminus allows formation of the proteolytically active form, cleavage within the central region of the propeptide allows formation of the mature double chain form. In the case of plant aspartic proteases, the PSS region is partially or totally removed from the precursor to produce the double chain form (Faro 1999).

There is some evidence for differential processing of the common enzyme precursor in different plant tissues, perhaps reflecting different physiological roles. For example the *H.vulgare* 48 kDa isoform was detected in all tissues whilst the 40 kDa isoform was not found in root or shoot (Martilla *et al.* 1995). Although the processing enzymes involved have not been characterised in plants there is evidence for auto-processing of some mammalian aspartic proteases (Mutlu and Gal 1999). In plants, processing of a

C.cardunculus precursor aspartic protease by an extract of *C.cardunculus* pistils which could be inhibited by pepstatin A has been described (Ramalho–Santos *et al.* 1998). The isolated protease was able to process a peptide representing one but not all cleavage sites required for maturation of its cognate pre-proprotein suggesting a role for auto-processing and/or processing by other protease isoforms.

A role for aspartic proteases related to cathepsin D in both protein processing and protein degradation has been demonstrated - for example enzyme precursor processing in yeast and animal cells (Rawlings and Bartlett 1995, D'Hondt *et al.* 1997), and degradation of oxidatively modified proteins such as superoxide dismutase in cardiac tissue extracts (Strack *et al.* 1996). Possible similar roles in seed-storage-protein precursor processing as well as hydrolysis of seed storage proteins in plants have been suggested (D'Hondt *et al.* 1997, Mutlu and Gal 1999). More intriguingly with regard to PPD, are recent results indicating a possible role of aspartic proteases in protein degradation and processing during senescence and cell death in both plant and mammalian systems.

In mammalian systems, proteases – specifically caspases of the cysteine protease family- have long been recognised as positive mediators of apoptosis. More recently however, cathepsin D, a lysosomal aspartic protease has been identified as a DAP (Death Associated Protein) gene in HeLa cells (Kissil and Kimchi 1998, Deis *et al.* 1996) and has been added to the list of proteases which function as positive mediators of apoptosis. The death promoting activity of cathepsin D was dependant on its protease activity since pepstatin A could protect cells from IFN-γ and Fas/APO-1 induced cell death; in addition, trasfection with a cathepsin D antisense cDNA exerted a similar protective effect [1]. Cathepsin D was found to exhibit an altered regulatory pattern during apoptosis at both the transcriptional and translational level. Levels of cathepsin D mRNA were elevated 3-4 fold after treatment with IFN-γ; whilst western blotting revealed altered processing of the protease. Normally, the inactive pre-protein is cleaved initially to a proteolytically active 48 kDa form and then to a mature double chain form of 14 – 30 kDa. After treatment with IFN-γ the intermediate 48 kDa form was found to accumulate and predominate in apoptotic cells at the expense of the mature double chain form, with a concomitant change in localisation from the lysosome to pre-

[1] IFN-γ is a cytokine belonging to the IFN (interferon) family of small secreted proteins. Binding of IFN-γ to its cognate cell surface receptor activates early signalling pathways leading to inhibition of cell proliferation and cell death. Fas/APO-1 is a cell membrane receptor for the TNF (tumour necrosis factor) family of cytokines. Initiation of signalling through Fas/APO-1 induces apoptosis in several mammalian cell types

lysosomal vesicles. This regulatory and subcellular localisation change is thought to be crucial for its apoptotic role. In a more recent follow-up study (Ollinger 2000), it was shown that inhibition of the aspartic protease cathepsin D could prevent free radical induced apoptosis in rat cardiomyocytes. Treatment of rat cardiomyocyte cells with the quinone free radical, naphthazarin, led to destabilization of lysosomal membranes followed by release of cathepsin D to the cytosol. Subsequently, activation of caspase-3-like protease activity and apoptotic cell morphology was observed. Pre-treatment of cells with the specific aspartic protease inhibitor, pepstatin A, did not prevent release of cathepsin D from lysosmes, but did significantly inhibit subsequent naphtharizin-induced caspase activity and apoptotic morphology. Pre-incubation of the cells with the free radical scavenger α-tocopherol inhibited both the release of cathepsin D and apoptotic cell death. Therefore, authors concluded that the aspartic protease cathepsin D exerts an apoptosis-stimulating effect upstream of caspase-3 activation.

In plants, aspartic protease activity has been described in senescent leaves of orange (*Citrus sinensis*) (Garcia-Martinez and Moreno 1986); senescent leaves of barley (*Hordeum vulgare*) (Kervinen *et al.* 1990); and senescing flowers and leaves of cardoon (*Cynara cardunculus*) (Heimgartner *et al.* 1990, Buchannan-Wollaston 1997). Recently, the barley aspartic protease phytepsin has been localised to developing tracheary elements and sieve cells, suggesting a role in autolysis during formation of tissues undergoing programmed cell death (Roonberg Roos and Saarma 1998). An aspartic protease like gene, nucellin, has also been shown to be specifically expressed in barley nucellar cells during developmentally programmed degeneration (Chen and Foolad 1997). Petals of the daylily (*Hemerocallis*) have been used as a model system for the study of organ senescence and PCD in plants, since they show a uniform senescence process over a 24 hour period without the need for an external stimulus. An aspartic protease has recently been identified as one of 6 DSA (Daylily Senescence Associated) genes in *Hemerocallis* (Panavas *et al.* 1999) and the authors propose that the aspartic protease may contribute directly to cell death via hydrolysis of cell components and may in addition activate other proteases. Likewise, a subtractive hybridisation approach led to the identification of an aspartic protease LSC760 as one of several senescence-related genes in *B.napus* (Buchanan-Wollaston and Ainsworth 1997).

To date, aspartic protease genes or cDNAs have been isolated from 14 plant systems including *Arabidopsis thaliana* (thale cress), *Lycopersicon esculentum* (tomato), *H.vulgare* (barley), *Oryzae sativa* (rice), *C.cardunculus* (cardoon), *B.napus* (oilseed rape), *Hemerocallis* (daylily), *Helianthus annuus* (sunflower), *Cicer arietinum*

(chickpea), *Vigna unguiculata* (cowpea), *Centaurea calcitrapa* (star thistle), *Cucurbita pepo* (pumpkin), *Helianthus annuus* (sunflower), *Pyrus pyrifolia* (pear), and *Brassica oleracea* (wild cabbage). However, several of these are partial sequences and limited further analysis has been carried out. Southern blot experiments indicate they may be encoded by a single gene as in *Arabidopsis* or by a small gene family as in *B.napus*. Characterisation and expression studies have been limited however, and considerable further research will be required to elucidate the precise role(s) and functioning of plant aspartic proteases *in vivo*.

The sequencing, characterisation and expression of a cassava aspartic protease MecASP1 during the post harvest period in cassava storage roots is described in section 7.2.1.

7.1.3 Cysteine proteases and cysteine protease inhibitors (cystatins)

The cysteine protease inhibitors or cystatins are protein inhibitors of cysteine proteases which inhibit the proteolytic activity of cysteine proteases via tight and reversible binding.

The cysteine proteases, particularly the caspase subfamily of cysteine proteases have been widely demonstrated to play an important role in the promotion of apoptosis. In animal cells such programmed cell death (PCD) may be triggered *via* 2 distinct but overlapping mechanisms – death-receptor mediated apoptosis, in which death receptors such as Fas/APO-1 located in the plasma membrane are triggered by their corresponding death ligands resulting in rapid activation of caspases; and stress or chemical induced apoptosis which is thought to involve perturbation of mitochondria and consequent release of cytochrome c from the mitochondrial intermembrane space, again resulting in a caspase activation cascade (reviewed in Cohen 2000, Song and Stellar 1999).

In plants elevation of cysteine protease activity appears to be a common result of environmental stresses and wounding (Linthorst *et al.* 1993, Williams *et al.* 1994, Lidgett *et al.* 1995, Stroeher *et al.* 1997, Jones and Mullet 1995). Although no homologues of the mammalian caspase subfamily have been isolated from plants (Yano *et al.* 1999) and to date a protease cascade has not been described (Schmid *et al.* 1999), there are numerous examples of plant cysteine proteases which have been identified as PCD/senescence associated and which are up regulated and/or specifically expressed in cells undergoing PCD. Examples include SAG2 and SAG12 of *Arabidopsis* leaves, See1 and See2 of *Zea mays* leaves, LSC7 and LSC790 of *B.napus* leaves, SEN10 of

Hemerocallis petals, pDCCP1 of *Dianthus caryophyllus* petals and PsCyp1 of *Pisum sativum* nodules (Lohman *et al.* 1994, Smart *el al* 1995, Buchanan- Wollaston 1997, Valpuesta *et al.* 1995, Jones *et al.* 1995 Kardailsky and Brewin 1996). In addition, activation of cysteine protease activity has been shown in cowpea (*Vigna unguiculata*) leaves undergoing HR (hypersensitive response) – a type of PCD; with some of the induced cysteine protease activity being similar to caspase activity in showing specificity for Asp (D) residues (D'Silva *et al.* 1998).

Cysteine protease inhibitors or cystatins have been described in both plant and animal systems. In plants 3 major roles – based on their inhibition of either exogenous or endogenous cysteine protease activity – have been proposed.

Since plant cystatins are commonly induced by wounding and methyl jasmonate, and are effective inhibitors of exogenous cysteine proteases, it has been suggested that they play an important role in plant defence against insect herbivory (Botella 1996, Pernas 1998, Alarcon and Malone 1995). In particular, in several species of the Hemidiptera and Coleoptera orders, cysteine proteases account for the majority of proteolytic activity responsible for protein digestion in the gut (Michaud *et al.* 1993). *In vitro* inhibition of digestive proteases, as well as *in vivo* effects on larval development have been demonstrated. In addition transgenic plants expressing rice cystatin have shown enhanced resistance to insect and nematode pests (Pernas *et al.* 1998). Recently, antifungal activity of a cysteine protease inhibitor isolated from pearl millet seeds has been reported (Joshi *et al.* 1998), with the inhibitor showing activity against several phytopathogenic fungi including *Claviceps, Helminthosporium, Alternaria* and *Fusarium* species.

With regard to inhibition of endogenous cysteine protease activity, possible roles in the modulation of cysteine proteases during seed maturation and germination, and PCD have been suggested. Since cysteine as well as other proteases may be involved in mobilisation of amino acids from storage proteins in seeds and propagative tubers it has been proposed that phytocystatins may regulate the action of cysteine proteases during seed maturation and germination - developmental stages when proteins must be accumulated for storage and later hydrolysed to amino acids (Botella *et al.* 1996). Recently, Solomon *et al.* (1999) have proposed cystatins as modulators of PCD in plants. In cultured soybean cells, oxidative stress induced PCD (H_2O_2 treatment, 5mM) involved activation of cysteine protease activity. When cells were transformed with constructs of 3 different protease inhibitors under control of the constitutive 35S CaMV promotor, the cystatin construct alone could inhibit both oxidative stress and pathogen

induced PCD. The authors thus suggest that plant PCD may be regulated at least in part by interactions between cysteine proteases and their cognate inhibitors.

The cystatin superfamily is further subdivided into 3 subfamilies- the family 1 cystatins or stefins, the family 3 cystatins or kininogens, and the rather confusingly named family 2 cystatins or cystatins (Turk and Bode 1991, Ryan *et al.* 1998). The plant cystatins, although homologous to family 1 cystatins in the absence of disulphide bonds show greater overall sequence similarity to the family 2 cystatins, and it has been proposed that the plant cystatins should be classified as a new family – the phytocystatins (Kouzouma *et al.* 1996, Ryan *et al.* 1998).

Plant cystatins have several highly conserved structural features including:

i) A glycine residue in the N-terminus

ii) A QxVxG motif (where x represents any residue) in the central portion of the protein.

iii) A proline-tryptophan (PW) motif in the C-terminus of the protein.

Bode *et al.* (1988).

In addition plant cystatins have been found to have highly conserved physio-chemical properties. Most monomeric cystatins have molecular weights of between 10 and 12 kDa, do not possess the disulphide bridge found in animal family 1 cystatins and generally have isoelectric points (*p*I) ≤ 7 (Kouzuma *et al.* 1996).

To date cysteine protease inhibitor amino acid sequences are available for 20 plant sytems including cowpea (*V.unguiculata*), soybean (*Glycine max*), chestnut (*Castanea sativa*), clove pink (*Dianthus caryophyllus*), sunflower (*H.annuus*), tomato (*L.esculentum*), papaya (*Carica papaya*), maize (*Z.mays*), sorghum (*Sorghum bicolour*), ragweed (*Ambrosia artemisiifolia*), mugwort (*Artemisia vulgaris*), pear (*Pyrus communis*), avocado (*Persea americana*), thale cress (*A.thaliana*), potato (*Solanum tuberosum*), cucumber (*Cucumis sativus*), rice (*O.sativa*), field mustard (*Brassica campestris*), sweet potato (*Ipombea batatas*) and castor bean (*Ricinus communis*). A large number of the protein sequences have been determined by protein chemical methods and a smaller number of genes or cDNAs have been cloned. They are encoded by small gene families and individual isoforms may show differential expression. For example, of 3 cysteine protease inhibitors, L1, R1 and N2, isolated from soybean, L1 showed constitutive expression in leaves, whilst R1 and N2 were induced following wounding or methyl jasmonate treatment (Botella *et al.* 1996).

The sequencing, characterisation and expression of a cassava cystatin MecCPI1 during the post harvest period in cassava storage roots is described in section 7.2.2.

7.1.4 Serine proteases

Leucine aminopeptidases of the serine protease class have been implicated in PCD in both plant and animal systems (Villa *et al.* 1997, D'Silva *et al.* 1998). Transcriptional activation of a serine protease has been described in xylogenesis of *Zinnia* cells, a process involving generation of H_2O_2 and cell death (Ye and Varner 1996). Similarly 3 serine protease activities have been described in senescent pea leaves (Distephano *et al.* 1997). Degradation of peroxisomal proteins in senescent pea leaves could be at least partially inhibited by phenylmethyl sulphony lfluoride (PMSF), an inhibitor of serine proteases (Distephano *et al.* 1999). However, in cultured soybean cells undergoing oxidative-stress induced PCD, tranformation with a serine protease inhibitor construct under control of the constitutive 35S CaMV promotor did not inhibit either oxidative stress or pathogen induced PCD (Solomon *et al.* 1999).

During the course of this project a cassava serine protease clone MecSER was obtained. The PCR product was initially sequenced, however the cDNA was not subcloned and no further analysis was carried out. For this reason the sequence has not been deposited at the NCBI database and the PCR sequence data only is presented in section 7.2.3

7.1.5 Cassava clones associated with transcription or translation

7.1.5.1 Translation initiation factor eIF-5A (formerly eIF4D)

A key regulatory step in translation is initiation, i.e. the recruitment of the translational machinery to the 5' end of the mRNA. Initiation of translation in eukaryotic cells is precisely regulated and in most cases is the rate-limiting step in protein synthesis (Raught and Gingras 1999). The eukaryotic translation initiation factors (eIFs) participate in the assembly of the 80s ribosome-mRNA complex, allowing the anti-codon of the initiator Met-tRNA to interact with the "start" AUG codon of the mRNA (figure 7.1.2). eIF-5A (eIF4D) is one of at least 5-6 factors involved in the initiation step of eukaryotic protein synthesis, although its precise role *in vivo* has not been as well characterised as that of other eIFs such as eIF2 (for review see Kozak *et al.* 1999). Although it has been shown in yeast that eIF-5A is not a prerequisite for translation (Kang and Hershey 1994) it has been identified in all eukaryotes examined and is one of the more abundant initiation factors in eukaryotic cells. It is conserved as a 16-18 kDa protein with an acidic pI of 5-6 (Chamot and Kuhlemeier 1992) and shows a high

degree of conservation with a close structural homologue in the Archae (Kyripedes and Woese 1998). To date it is the only known cellular protein containing the unusual amino acid hypusine [(4-amino-2-hydroxybutyl) lysine] formed via a post-translational modification of a conserved K residue.

Proposed roles for eIF-5A include stabilisation of the charged Met-tRNA in the 80s ribosome-mRNA complex, and possible roles in mRNA export (Kang and Hershey 1994). Multiple forms of eIF-5A have been described in some species and it has been suggested the factor fulfils other functions in addition to its role in translation initiation (Dresselhaus *et al.* 1999). In tobacco (*Nicotinia plumbaginifolia*) differential expression of 2 eIF-5A isoforms was observed (Chamot and Kuhlemier 1991) with 1 isoform expressed constitutively whilst the second was expressed predominantly in photosynthetically active tissues. In maize (*Zea mays*) eIF-5A expression was found mainly in metabolically active cells (Dresselhaus *et al.* 1999, Chamot and Kuhlemier 1992). This differential regulation suggests that eIF-5A may play a key role in regulation of the initiation step of translation, and is responsive to the metabolic state of the cell. In addition, it has been proposed that eIF-5A could function in mediating regulation of translation in response to developmental and stress responses. The lysine (K) to hypusine modification has been shown to be essential for eIF-5A activity *in vitro* and this modification requires the polyamine spermine. Plant polyamines have been implicated in a variety of developmental and stress responses and it is proposed that eIF-5A may represent the mechanism through which these responses are effected, since depletion of cellular polyamines would suppress formation of the critical hypusine modification (Chamot and Kuhlemier 1992, Tome and Gerner 1997).

Sequencing, sequence analysis and southern analysis of a cassava translation initiation factor MecTIF is described in section 7.3.1.

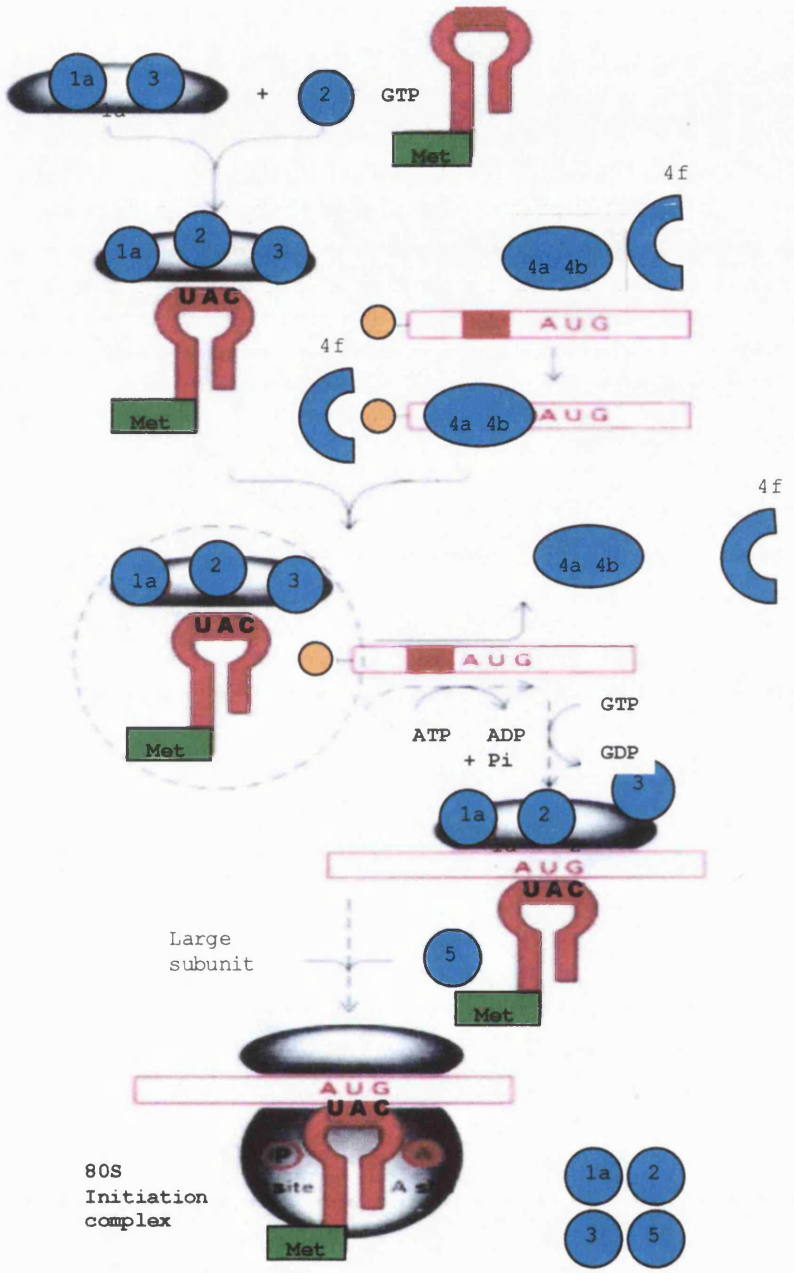


Figure 7.1.2 Schematic model of eukaryotic translation initiation. Initiation factor eIF2 binds a molecule of GTP and the Met-tRNA^{Met} forming the ternary complex. The complex binds to the small 40S ribosomal subunit as well as other initiation factors such as eIF3 and eIF1a, allowing activation of the small subunit complex. The 5'end of the mRNA associates with several proteins including eIF4 which are thought to be involved in recognition of the 5' cap and unwinding of secondary RNA structure at the 5' end. The 40S initiation complex associates with the 5' end of the mRNA and the Met-tRNA^{Met} is transferred to its correct position at the start AUG codon of the mRNA. The large 60S ribosomal subunit then binds to the complex via the ribosome binding site on the mRNA forming the 80S initiation complex. Initiation factors are shown in blue, the mRNA cap is shown in orange.

7.1.5.2 RNA polymerase subunit RPB8

In prokaryotes, RNA is transcribed by a single polymerase, however eukaryotes in contrast contain 3 discrete RNA polymerases (Roeder and Rutter 1969) differing in template specificity, localisation and susceptibility to the inhibitor α-amanitin (figure 7.1.3). All 3 polymerases are large multi-subunit enzymes of 500 kDa or more, typically containing 8-14 subunits, which require additional proteins - termed general transcription factors - in order to form an active transcription complex which can recognise the promoter and initiate transcription. The study of subunit roles and composition for eukaryotic RNA polymerases remains at an early stage. To date, most research has been directed at the yeast (*S.cerevisiae*) pol II enzyme, which contains 12 subunits designated RPB1-12. Five of these subunits (RPB5, RPB6, RPB8, RPB10, and RPB12) are also present in pol I and pol III, and therefore represent common subunits (McKune *et al.* 1995). As yet, RNA polymerase II is poorly understood at the biochemical level and the function of many of the subunits, including RPB8, remains unclear. Their role in transcription has been studied mainly by isolating the genes responsible and carrying out substitutions with yeast homologues; often, the heterologous units are fully functional suggesting high structural homology between the proteins (McKune *et al.* 1995). To date only a single plant homologue (from *Arabidopsis*) of RPB8 has been identified (Larkin and Guilfoyle, unpublished).

In section 7.3.2 the sequence, sequence analysis and southern analysis of a cassava homologue of RPB8 – MecRPB8 is described.

RNA polymerase	Location	Transcribes	Sensitivity to α-amanitin
pol I	nucleolus	18s, 5.8s and 28s rRNA	insensitive
pol II	nucleoplasm	mRNA and snRNAs	highly sensitive
pol III	nucleoplasm	tRNA, U6 snRNA and 5s rRNA	Sensitive to high concentrations

Figure 7.1.3 The eukaryotic RNA polymerases

7.1.6 Chapter summary

Sequence data for 5 cassava cDNA clones – MecASP1, MecCPI1, MecSER1, MecTIF1 and MecRPB8 – is presented. Deduced amino acid sequences and sequence analysis of all 5 clones are discussed. Results of southern analyses are discussed for MecASP1, MecCPI1, MecTIF1 and MecRPB8. Transcript expression patterns of MecASP1 and MecCPI1 in cassava storage roots during the post harvest period are described.

7.2 Characterisation and expression of proteases and protease inhibitors expressed during cassava PPD

7.2.1 Cassava aspartic protease MecASP1

The sequence and deduced translation of a partial cassava aspartic protease with homology to mammalian cathepsin D is shown in figure 7.2.1.2. The clone was designated MecASP1 (*Manihot esculenta* cDNA encoding Aspartic protease 1) and has been submitted to the Genbank database under the accession number AF266465. The clone is 710 bp in size and encodes a predicted protein of 159 amino acids. The sequencing strategy used is shown below (figure 7.2.1.1)

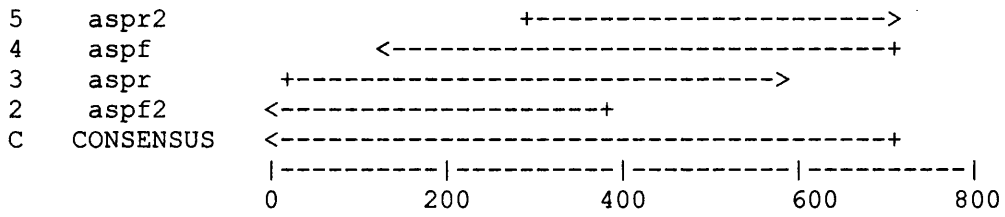


Figure 7.2.1.1 GELASSEMBLE output showing sequencing strategy for MecASP1. Universal M13/pUC primers were used for initial forward and reverse sequencing reactions. For subsequent internal sequencing reactions oligonucleotide primers were designed based on sequence data obtained, using the "Primer Designer" software programme.

Comparison of the cassava sequence with that of other plant aspartic protease sequences indicate that MecASP1 is a partial clone. The cDNA is truncated at the 5' end and comprises sequences encoding the central plant specific sequence (PSS) and the C terminal region of the pre-proprotein. Characteristic features described for the plant aspartic protease PSS region include:

- the presence of 6 conserved Cys residues
- a single conserved glycosylation site
- sequence and topological homology to mammalian saposins and saposin like proteins such as NK lysin

(Mutlu and Gal 1999, Kervinen *et al.* 1999).

Since saposins and saposin-like proteins are known to associate with membrane lipids and membrane bound proteins it has been proposed that the PSS of plant aspartic proteases may play a role in vacuolar targeting of the pre-proprotein via promotion of interaction with membrane receptor proteins during Golgi mediated transport to the vacuole (Kervinen *et al.* 1999). The location of these features within the deduced amino

acid sequence of MecASP1 are shown in figure 7.2.1.2. The conserved N-glycosylation site (N-A-T) is found at residues 50-53 of the deduced translation presented here. A second putative glycosylation site (N-V-S) occurs at residues 86-88. As may be noted only 4 of the 6 conserved Cys residues are present - at positions 1, 33, 36 and 64 – indicating that the PSS region of this clone is itself somewhat truncated at the 5' end. Analysis of the sequence using the ProfileScan proteomics tool on the ExPASy (Expert Protein Analysis System) website (http://expasy.cbr.nrc.ca) indicated a saposin B type

```
1     gtgcacattcgatggttctcgaggtgtaagtatgacaattgagagtgttgtgaatgagaa
        C  T  F  D  G  S  R  G  V  S  M  T  I  E  S  V  V  N  E
61    tagtcaagaagtggctggtagtttgcatgatgcaatgtgctctacttgtgagatggcagt
      N  S  Q  E  V  A  G  S  L  H  D  A  M  C  S  T  C  E  M  A
121   catttggatgcagaatcaactcaagcagaatgcaacactggagcgcatacttaactatgc
      V  I  W  M  Q  N  Q  L  K  Q  N  A  T  L  E  R  I  L  N  Y
181   caatgagctatgtgaacgattgcctagtccaatgggggaatcagccgtagattgtggtag
      A  N  E  L  C  E  R  L  P  S  P  M  G  E  S  A  V  D  C  G
241   cttgtctaccatgcctaacgtttcatttacaattggcgggaaggtttttgatctctcccc
      S  L  S  T  M  P  N  V  S  F  T  I  G  G  K  V  F  D  L  S
301   tgagcagtatgtactcaaagtgggtgagggagaagctgctcagtgcattagtggatttac
      P  E  Q  Y  V  L  K  V  G  E  G  E  A  A  Q  C  I  S  G  F
361   agctctagatgtgccaccactcgtgggcccctctggatactgggagatgttttcatggg
      T  A  L  D  V  P  P  P  R  G  P  L  W  I  L  G  D  V  F  M
421   tcggttccatacagtattcgactatggtaatctgagagttggatttgcggaagctgcata
      G  R  F  H  T  V  F  D  Y  G  N  L  R  V  G  F  A  E  A  A
481   acctttactacgttgttctgttttgtgttgtacaccttgaccttgctccatctgtatgc
541   ttttacgtgaaagtgtcgttgttgactttgaagggcttgaactaaacctcgtgtaaata
601   tcagaacagccttgttatgttgtgaaactagtactgaaccttcactgcataaccttacta
661   cgtagttctgttgtgttgtgcaaaaaaaaaaaaaaaaaaaaaaaaaaaa
```

Figure 7.2.1.2 Nucleotide sequence and deduced translation of the cassava aspartic protease clone MecASP1. Nucleotide sequence features – the predicted stop codon and three putative polyadenylation signals are shown in red. Within the deduced polypeptide sequence amino acids in turquoise represent the ProSite eukaryotic aspartic proteases active site motif (PS00128). Grey amino acids show a saposin type B domain. Conserved Cys residues of the PSS are shown in bold red. The a conserved N-glycoslyation motif (N-A-T) and a second putative glycosylation site (N-V-S) are shown in pink.

domain located from residue 29-70. In addition, the ProfileScan analysis revealed a eukaryotic aspartic protease active site motif, approaching the consensus [LIVMFGAC]-[LIVMTADN]-[LIVFSA]-D-[ST]-G-[STAV][STAPDENQ]-x-[LIVMFSTNC]-x-[LIVMFGTA] where D is the active site residue. The sequence did not contain either of the exact DTG or DSG active site motifs however, confirming that MecASP1 contains the C-terminal segment of the pre-pro-protein. The C terminal part, although highly similar to the N-terminal segment, does not contain the active aspartyl residues (Mutlu and Gal 1999).

The nucleotide sequence terminates with an ochre termination signal (TAA) at position 479. The 3' UTR contains no consensus polyadenylation signal (AATAAA) although 3 sequences approaching the consensus are found which may serve this function *in vivo*.

In a blastx search on the NCBI database (www.ncbi.nlm.nih.gov), the cassava sequence showed highest similarity to the *Cucurbita pepo* aspartic protease over the region compared (77% amino acid identity), similarity to human cathepsin D was 54% identity. A multiple alignment of the MecASP1 deduced amino acid sequence with those of other similar plant aspartic proteases as well as mammalian cathepsin D sequences, was constructed using ClustalW within gcg (Devereux *et al* 1984). The C terminal part of this alignment is shown in figure 7.2.1.3 (please see appendix C for the full alignment). The modular structure of aspartic proteases and the absence of sequences related to the PSS in mammalian cathepsin D is clearly evident; as is the unusual structure of barley nucellin (Chen and Foolad 1997) which lacks both the propeptide and the PSS. It is of note that the conserved glycosylation site within the PSS is strongly, but not entirely, conserved and is not present in the *C.arietum* or *C.cardunculus* preprocardosin A sequences. This conserved glycosylation site has been experimentally demonstrated to be modified in the barley nucellin 16kDa subunit and such glycosylation may be related to function (Lindholm *et al.* 2000).

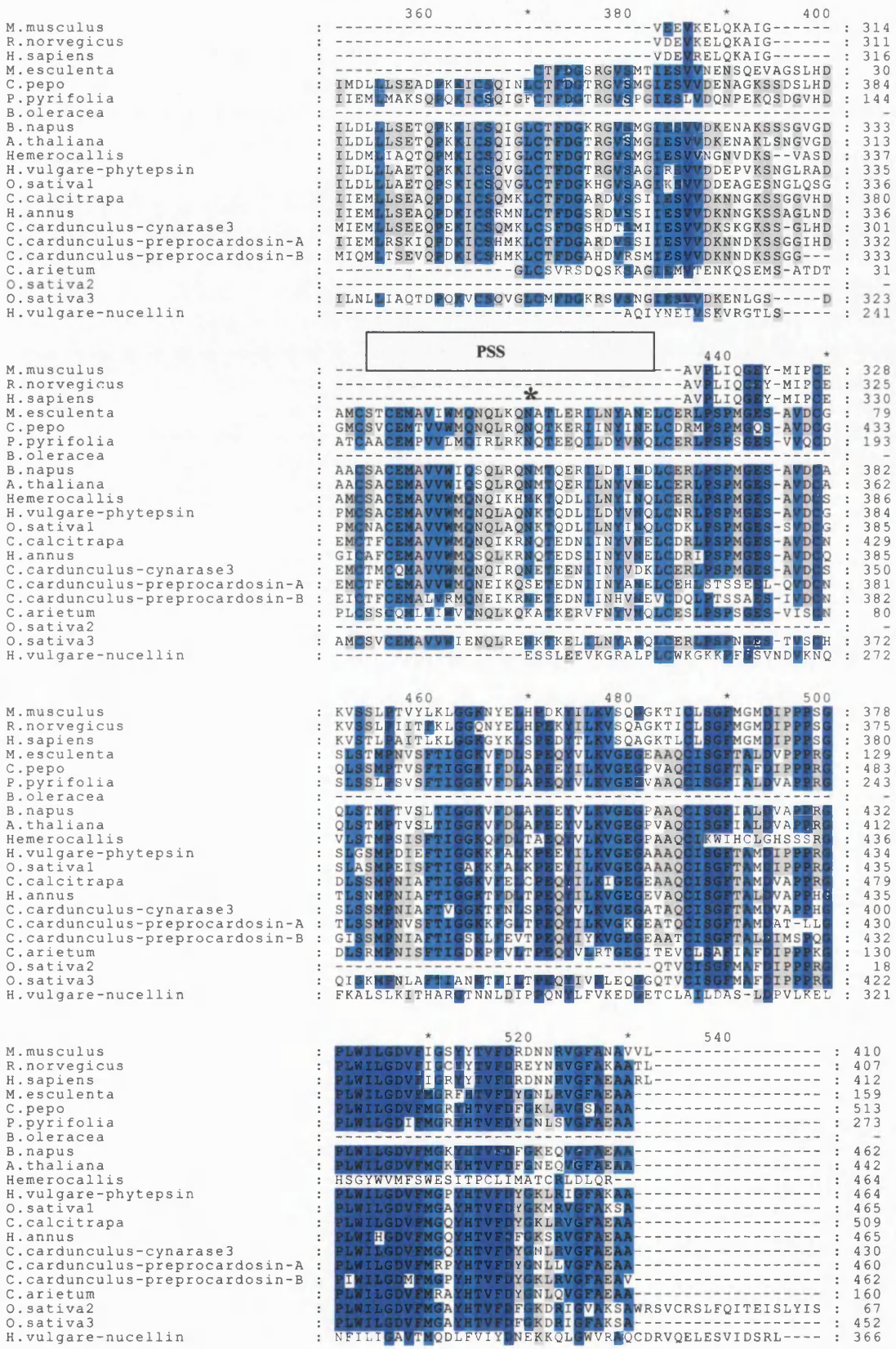


Figure 7.2.1.3 Amino acid alignment of plant and animal aspartic protease sequences. The C terminal part of the alignment only is shown here. Conserved residues are shown in colour blocking. Blue indicates >80% conservation, cyan indicates > 60% conservation and grey indicates > 40% conservation. The plant specific sequence (PSS) is shown boxed. The conserved glycosylation motif (S/N-x-T) is indicated by an asterisk.

In order to provide initial information on whether the cassava aspartic protease encoded by MecASP1 represented a "housekeeping" aspartic protease or a protease which could potentially be involved in PPD of cassava storage roots, the question of whether its regulation was altered during the course of PPD was addressed. Cassava storage roots for these experiments were freshly harvested from the field at CIAT, Colombia, and were then injured by removal of the proximal and distal ends of the roots and cutting of 2 "V" shaped incisions through the epidermis along the length of the root. The root ends were covered with parafilm and roots were stored in an open-air shed. For northern blotting experiments total RNA was extracted daily from storage roots over a 5 day time course. Under these storage conditions visible symptoms of PPD occurred within 24 hours after harvest and the PPD response progressed rapidly. Northern blotting and hybridisation procedures were carried out as described in section 2.7.10 using MecASP1 as the probe. Intriguingly the transcript showed up regulation during the post harvest storage period with an increase within 24 hours (1 day) and approximately 3-4 fold induction by 2 to 3 days after harvest. As discussed in section 7.1.2 expression studies on plant aspartic proteases have not been extensive to date, however similar transcript up regulation has been described during senescence or programmed cell death responses in petals of *Hemerocallis* (Panavas *et al.* 1999), senescent leaves of *B.napus* (Buchanan-Wollaston and Ainsworth 1997), degenerating nucellar cells of *H.vulgare* embryo (Chen and Foolad 1997), and in wounded leaves of *L.esculentum* (Schaller and Ryan 1996). In several instances, for example the *H.vulgare* phytepsin and nucellin, expression of the aspartic protease is highly localised to particular organs or cell types. Further analysis of the expression of MecASP1 and its cognate protease using northern analysis of different tissue types and immunohistochemical or *in situ* approaches would be of interest.

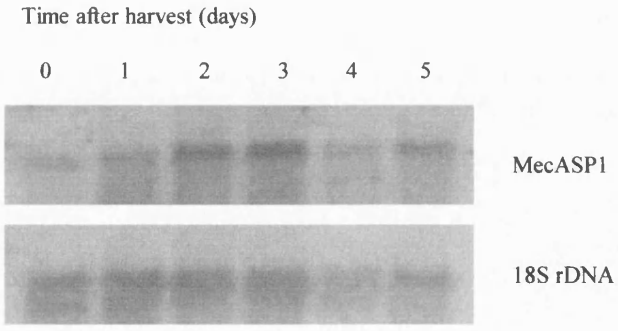


Figure 7.2.1.4 mRNA transcript accumulation of MecASP1 in cassava storage roots (cultivar MDOM5) during post-harvest physiological deterioration. 10µg total RNA isolated from storage roots at various times after harvest were electrophoresed on a denaturing formaldehyde gel and northern blotted according to standard procedures (Sambrook *et al.* 1989). As a control for equal loading the same blot was stripped and re-hybridised with an 18S rDNA probe (lower panel).

To investigate MecASP1 gene organisation in cassava, Southern blotting experiments were carried out as described in section 2.7.9. The "Clonemanager" programme (Scientific and Educational Software, 1995) was used to generate a restriction map of MecASP1, and 4 restriction enzymes which did not cut within the cDNA sequence – *Bgl*II, *Eco*RI, *Eco*RV and *Hind*III – were selected for digestion of genomic DNA. Hybridisation was carried out overnight at 55° C using MecASP1 as probe. For the low stringency wash to allow detection of related sequences the following regime was used – two 30 minute washes in 2X SSC, 0.1% SDS at 55° C, followed by two 20 minute washes in 1X SSC, 0.1% SDS at 60° C. Following autoradiography, the membrane was re-washed at high stringency (two 20 minute washes in 0.1X SSC, 0.1% SDS at 60° C) to allow detection of the cognate gene only. Results are shown below (figure 7.1.2.5). Data from the high stringency wash (panel B) indicate that MecASP1 occurs in the cassava genome as a single copy gene, containing at least 1 *Hind*III restriction site. When compared with the low stringency wash (panel A), results indicate that there is at least one other related aspartic protease gene in the cassava genome (note, in particular, the strongly hybridising bands above 9.4 kb and 4.6 kb in lanes 2 and 4 respectively, which are not present after the high stringency wash). These results would be in agreement with published data indicating that plant aspartic proteases occur as small gene families. At least 2 genes have been reported for *H.vulgare* and *O.sativa*, whilst at least 4 have been reported for *B.napus* (Mutlu and Gal 1999, D'Hondt *et al.* 1997).

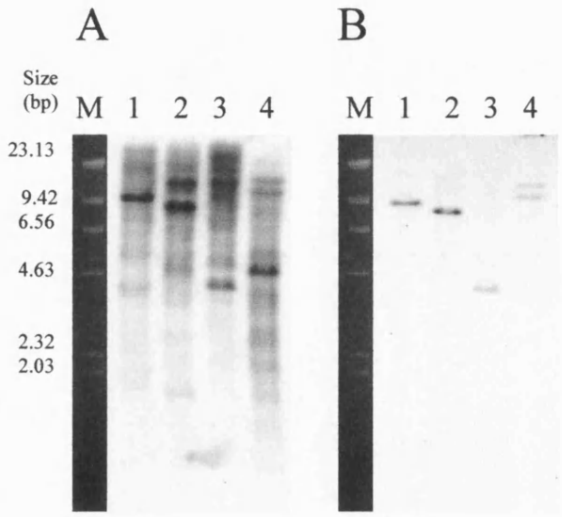


Figure 7.2.3.5 Southern blot analysis of *M. esculenta* nuclear gene organisation. Genomic DNA (20µg per lane) digested with the restriction enzymes *Bgl*II (lane 1) *Eco*RI (lane 2), *Eco*RV (lane 3) and *Hind*III (lane 4) and was electrophoresed at 1.5v cm^{-1} on a 0.8% TAE gel and Southern blotted according to standard procedures. The blot was hybridised with MecASP1 as a probe. Panel A = low stringency wash, panel B = high stringency wash.

7.2.2 Cassava cysteine protease inhibitor MecCPI1

The sequence and deduced translation of a cassava cysteine protease inhibitor with homology to other plant cystatins is shown in figure 7.2.2.3. The clone was designated MecCPI11 (*Manihot esculenta* cDNA encoding Cysteine protease inhibitor 1) and has been submitted to the Genbank database under the accession number AF265551 The clone is 480bp in size and encodes a predicted protein of 101 amino acids. The sequencing strategy used is shown below (figure 7.2.2.1)

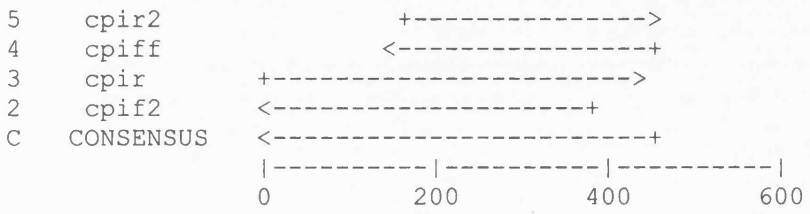


Figure 7.2.2.1 GELASSEMBLE output showing sequencing strategy for MecCPI1. Universal M13/pUC primers were used for initial forward and reverse sequencing reactions. For subsequent internal sequencing reactions oligonucleotide primers were designed based on sequence data obtained, using the "Primer Designer" software programme.

The clone was originally isolated as a chimeric cDNA (MecCPI/RPB8) containing sequences encoding RNA polymerase subunit RPB8 as well as cystatin sequences arranged in opposite orientation (as shown in figure 7.2.2.2) which is thought to have arisen by blunt end ligation of 2 cDNAs during library construction. In order to separate the 2 sequences the original clone was fully sequenced and a PCR based approach was used to amplify cystatin and RPB8 sequences as shown in figure 7.2.2.2. The discrete PCR products were then subcloned into pGEM-T easy vector. The sequence of MecCPI/RPB8 and internal primers used is given in appendix D.

In order to verify that no errors had been introduced during PCR amplification, the sequence of the novel constructs, MecCPI1 and MecRPB8, were compared to the original clone using the "blast 2 sequences" programme available on the NCBI homepage (http://www.ncbi.nlm.nih.gov).

The deduced protein sequence for MecCPI1 was analysed using the ExPASy (Expert Protein Analysis System) Proteomics website. Characteristic conserved motifs described in the cystatin literature:

- a conserved N terminal Gly (G) residue
- a conserved C terminal Pro-Trp (PW) motif
- an internal Gln-x-Val-x-Gly motif

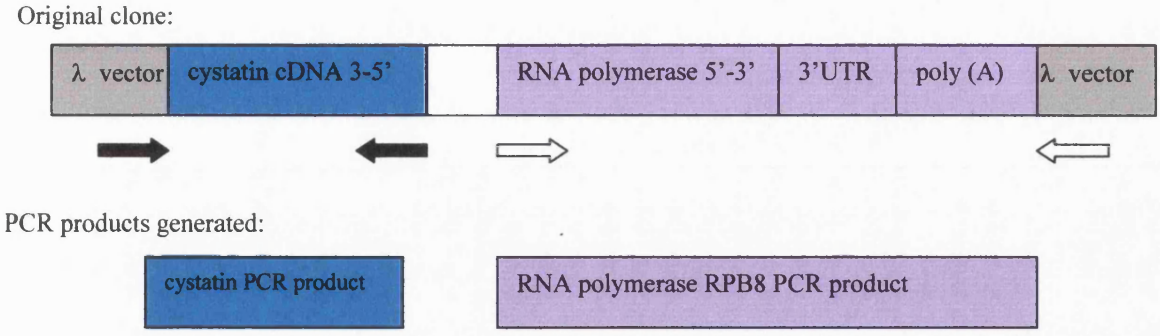


Figure 7.2.2.2 PCR strategy used for subcloning MecCPI1 and MecRPB8 from an original chimeric clone MecCPI/RPB8. Primer pair 1 (shown in black) consisted of the λ vector forward primer and an internal primer designed within the cystatin coding sequence. Primer pair 2 (shown in white) consisted of the λ vector reverse primer and an internal primer designed within the RNA polymerase coding sequence. PCR reactions were carried out using the original phage clone as template. PCR products of expected size were gel purified and ligated into pGEM-T easy in order to generate the MecCPI1 and MecRPB8 constructs.

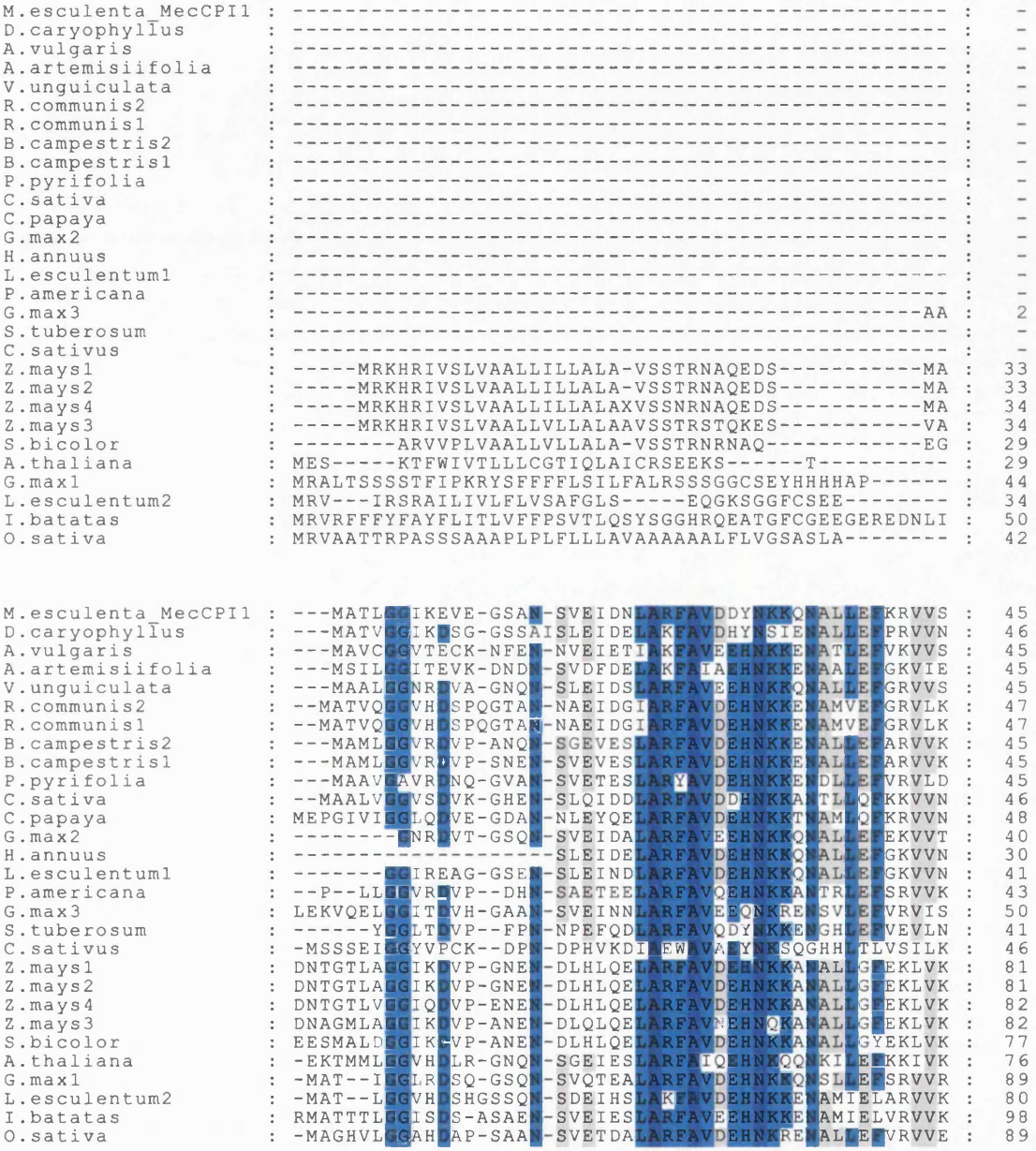
were present in the deduced polypeptide and are indicated in figure 7.2.2.3. In addition a sequence closely approaching the Prosite cysteine protease inhibitor consensus sequence motif (consensus [GSTE**Q**KRV]**Q**[LI**V**T][**V**AF][S**A**GQ]**G**x[LIV**M**NK][LIV MF**Y**][LIVMF**Y**]x[**L**IVMFYA][D**E**NQKRHSIV] was also present. The predicted molecular weight and isolelectric point of the deduced protein as calculated using the ProtParam programme (Bjellquist *et al.* 1994) on the ExPASy website (http://expasy.cbr.nrc.ca/cgi-bin/protparam) were pI: 5.12 and 1.12k kDa. Both of these values, although hypothetical, fall within the expected range for plant cystatins (Kouzuma *et al.* 1996).

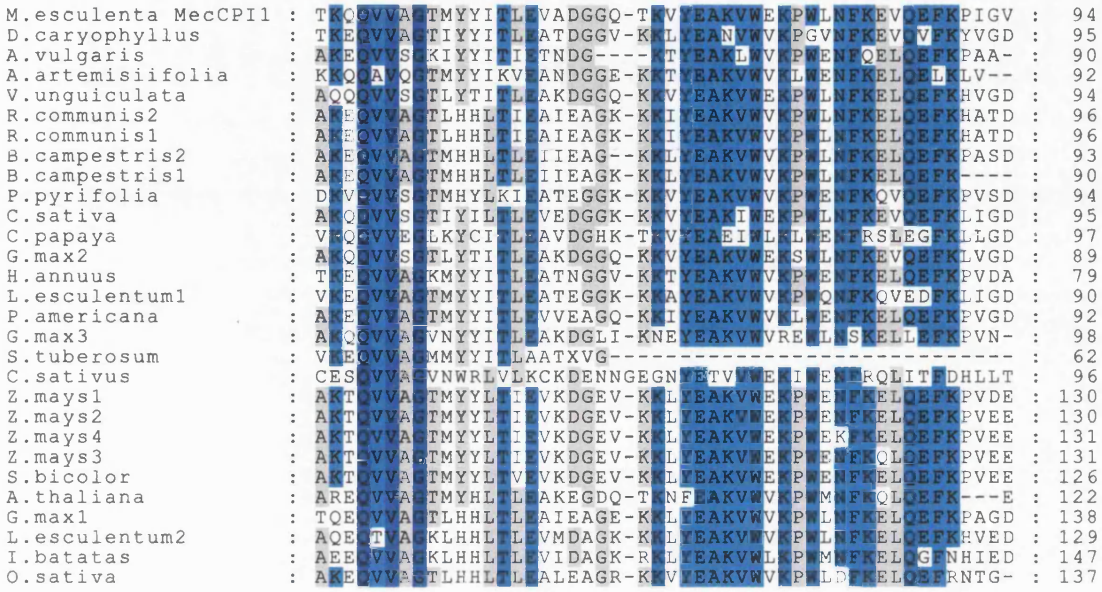
Analysis of the sequence using the PSORT series of programmes (www.psort.nibb.ac.jp) did not reveal the presence of any targeting sequences or motifs, and indicated that the protein may be cytoplasmic (P = 0.45) (Nakai, 2000). The nucleotide sequence contains an amber termination signal (TAG) located at position 306 and is followed by a short 3'UTR containing an exact consensus polyadenylation signal (AATAA) as well as a related AATAA motif. The ATG encoding the first methionine in the sequence presented here, lies within a motif approaching the Kozak consensus for eukaryotic translation initiation. Purines (A or G) are present in the –3 and +4 positions (Kozak 1986, Joshi 1997) suggesting that the full coding sequence is present in the clone.

```
1     gaaatggcaactttaggaggtattaaggaagtggaggaatcggccaacagtgttgagatc
          M  A  T  L  G  G  I  K  E  V  E  E  S  A  N  S  V  E  I
61    gataacctagctcgtttcgccgtcgatgattacaacaagaaacagaatgcgttgctggag
        D  N  L  A  R  F  A  V  D  D  Y  N  K  K  Q  N  A  L  L  E
121   tttaagagagtggtgagtacaaagcagcaggtggtggctgggaccatgtactatattact
        F  K  R  V  V  S  T  K  Q  Q  V  V  A  G  T  M  Y  Y  I  T
181   ttggaggtagctgatggcggtcaaaccaaagtttatgaggccaaggtctgggaaaagcca
        L  E  V  A  D  G  G  Q  T  K  V  Y  E  A  K  V  W  E  K  P
241   tggttgaattttaaggaggtccaggaattcaagccaattggggttgccccttcagactct
      W  L  N  F  K  E  V  Q  E  F  K  P  I  G  V  A  P  S  D  S
301   accgcttaggtgactgaagacaatgtatatgctactgcgcttggagttgatgctcaaaat
        T  A  -
361   aaagagtaaagaataatgttgacagtattttatttcccttatctaactggaatcgtctat
421   atgaaatgggtatttatgaatggttacatctatcttatctgtgccatggtacccggatcc
```

Figure 7.2.2.3 Nucleotide sequence and deduced translation of the cassava cysteine protease inhibitor clone MecCPI1. Nucleotide sequence features – the predicted start and stop codons and two putative polyadenylation signals are shown in red. Protein sequence features reffered to in the text – the N terminal glycine (G), amino terminal proline-tryptophan (P-W) and internal Q-x-V-x-G motifs are shown in turquoise. Grey amino acids indicate the cysteine proteinase binding site.

An amino acid alignment of the predicted cassava sequence with those of other plant cystatins is shown in figure 7.2.2.4. Sequences used were cowpea (*V.unguiculata*), soybean (*Glycine max*), chestnut (*Castanea sativa*), clove pink (*Dianthus caryophyllus*), sunflower (*H.annuus*), tomato (*L.esculentum*), papaya (*Carica papaya*), maize (*Z.mays*), sorghum (*Sorghum bicolour*), ragweed (*Ambrosia artemisiifolia*), mugwort (*Artemisia vulgaris*), pear (*Pyrus communis*), avocado (*Persea americana*), arabidopsis, potato (*Solanum tuberosum*), cucumber (*Cucumis sativus*), rice (*O.sativa*), field mustard (*Brassica campestris*), sweet potato (*Ipombea batatas*) and castor bean (*Ricinus communis*).


M.esculenta_MecCPI1
D.caryophyllus
A.vulgaris
A.artemisiifolia
V.unguiculata
R.communis2
R.communis1
B.campestris2
B.campestris1
P.pyrifolia
C.sativa
C.papaya
G.max2
H.annuus
L.esculentum1
P.americana
G.max3
S.tuberosum
C.sativus
Z.mays1
Z.mays2
Z.mays4
Z.mays3
S.bicolor
A.thaliana
G.max1
L.esculentum2
I.batatas
O.sativa


M.esculenta MecCPI1 : TKQQVVAQTMYYITLEVADGGQ-TKVYEAKVWEKPWLNFKEVQEFKPIGV : 94
D.caryophyllus : TKEQVVAQTIYYITLEATDGGV-KKLYEANVWVKPGVNFKEVQVFKYVGD : 95
A.vulgaris : AKEQVVSQKIYYITIETNDG----KTYEAKLWVKPWENPQELQEFKPAA- : 90
A.artemisiifolia : KKQQAVQQTMYYIKVEANDGGE-KKTYEAKVWVKLWENFKELQELKLV-- : 92
V.unguiculata : AQQQVVSQTLYTITLEAKDGGQ-KKVYEAKVWEKPWLNFKELQEFKHVGD : 94
R.communis2 : AKEQVVAQTLHHLTIEAIEAGK-KKIYEAKVWVKPWLNFKELQEFKHATD : 96
R.communis1 : AKEQVVAQTLHHLTIEAIEAGK-KKIYEAKVWVKPWLNFKELQEFKHATD : 96
B.campestris2 : AKEQVVAQTMHHLTLEIIEAG--KKLYEAKVWVKPWLNFKELQEFKPASD : 93
B.campestris1 : AKEQVVAQTMHHLTLEIIEAGK-KKLYEAKVWVKPWLNFKELQEFK---- : 90
P.pyrifolia : DKVEQVVSQTMHYLKIEATEGGK-KKVYEAKVWVKPWENFKQVQEFKPVSD : 94
C.sativa : AKQQVVSQTIYILTLEVEDGGK-KKVYEAKIWEKPWLNFKEVQEFKLIGD : 95
C.papaya : VKQQVVEQLKYCITLEAVDGHK-TKVYEAEIWLKLWENFRSLEGFKLLGD : 97
G.max2 : AKQQVVSQTLYTITLEAKDGGQ-KKVYEAKVWEKSWLNFKEVQEFKLVGD : 89
H.annuus : TKEQVVAQKMYYITLEATNGGV-KKTYEAKVWVKPWENFKELQEFKPVDA : 79
L.esculentum1 : VKEQVVAQTMYYITLEATEGGK-KKAYEAKVWVKPWQNFKQVEDFKLIGD : 90
P.americana : AKEQVVAQTMYYITLEVVEAGQ-KKIYEAKVWVKLWENFKELQEFKPVGD : 92
G.max3 : AKQQVVAQVNYYITLEAKDGLI-KNEYEAKVWVRELLSKELLEFKPVN- : 98
S.tuberosum : VKEQVVAQMMYYITLAATXVG---------------------------- : 62
C.sativus : CESQVVAQVNWRLVLKCKDENNGEGNKETVTWEKIKEKFRQLITFDHLLT : 96
Z.mays1 : AKTQVVAQTMYYLTIEVKDGEV-KKLYEAKVWEKPWEKFKELQEFKPVDE : 130
Z.mays2 : AKTQVVAQTMYYLTIEVKDGEV-KKLYEAKVWEKPWEKFKELQEFKPVEE : 130
Z.mays4 : AKTQVVAQTMYYLTIEVKDGEV-KKLYEAKVWEKPWEKFKELQEFKPVEE : 131
Z.mays3 : AKTQVVAQTMYYLTIEVKDGEV-KKLYEAKVWEKPWEKFKQLQEFKPVEE : 131
S.bicolor : AKTQVVAQTMYYLTVEVKDGEV-KKLYEAKVWEKPWEKFKELQEFKPVEE : 126
A.thaliana : ARECVVAQTMYHLTLEAKEGDQ-TKNFEAKVWVKPWMKFKQLQEFK---E : 122
G.max1 : TQEQVVAQTLHHLTLEAIEAGE-KKLYEAKVWVKPWLNFKELQEFKPAGD : 138
L.esculentum2 : AQEQTVAQKLHHLTLEVMDAGK-KKLYEAKVWVKPWLNFKELQEFKHVED : 129
I.batatas : AEEQVVAQKLHHLTLEVIDAGK-RKLYEAKVWLKPWMNFKELQGFNHIED : 147
O.sativa : AKEQVVAQTLHHLTLEALEAGR-KKVYEAKVWVKPWLDFKELQEFRNTG- : 137


M.esculenta MecCPI1 : ----------APSDSTA---------- : 101
D.caryophyllus : ----------AFG---------- : 98
A.vulgaris : ---------- : -
A.artemisiifolia : ---------- : -
V.unguiculata : ----------APA---------- : 97
R.communis2 : VADTTASHPSFTSSDLGVKREGHGAEWKEVAAHDPVVQDAATHAVNTIQQ : 146
R.communis1 : VADTTASHPSFTSSDLGVKREGHGAEWKEVAAHDPVVQDAATHAVNTIQQ : 146
B.campestris2 : DG---SPSATITPSDLGCKKGEGASGWREVPGDDPEVQHVADHAVKTIQQ : 140
B.campestris1 : ------PSTTITPSDLGCKKGEGASGWREVPGDDPEVQHVADHAVKSIQQ : 134
P.pyrifolia : S---------- : 95
C.sativa : ----------APTHHSA---------- : 102
C.papaya : ----------AH---------- : 99
G.max2 : ----------APA---------- : 92
H.annuus : ----------ATS---------- : 82
L.esculentum1 : ----------AATA---------- : 94
P.americana : --------SSSTSSDA---------- : 100
G.max3 : ----------VSSTQ---------- : 103
S.tuberosum : ---------- : -
C.sativus : ---------- : -
Z.mays1 : ----------GASA---------- : 134
Z.mays2 : ----------GASA---------- : 134
Z.mays4 : ----------GASA---------- : 135
Z.mays3 : ----------GASA---------- : 135
S.bicolor : ----------GASA---------- : 130
A.thaliana : ----------SSS---------- : 125
G.max1 : V-------PSFTSADLGVKKDGHQPGWQSVPTHDPQVQDAANHAIKTIQQ : 181
L.esculentum2 : V-------PTFTSSDLGVKQVEQNSGLKSVPVHDPVVEEAAEHAIKTIQQ : 172
I.batatas : I-------PTLTSSDLGAKRDWPNTGLKSVPVNDPVVQEAAQHAVKTIQQ : 190
O.sativa : ------DATTFTNADLGAKKGGHEPGWRDVPVHDPVVKDAADHAVKSIQQ : 181

```
M.esculenta MecCPI1 : ------------------------------------------------- :   -
D.caryophyllus      : ------------------------------------------------- :   -
A.vulgaris          : ------------------------------------------------- :   -
A.artemisiifolia    : ------------------------------------------------- :   -
V.unguiculata       : ------------------------------------------------- :   -
R.communis2         : RSNSLFPYQLQEIVHAKAQVVDDFAKFDMILKVKRGTSG-R---EVQG-- : 190
R.communis1         : RSNSLFPYQLQEIVHAKAQVVDDFAKFDMILKVKRGTSEEKFKVEVHKNN : 196
B.campestris2       : RSNSLFPYELQEVVHANAEVTGEAAKYNMVLKLKRGEKEEKFKVEVHKNH : 190
B.campestris1       : RSNSLFPYELQEVVHANAEVTGEAAKYNMVLKLKRGEKEEKFKVEVHKNH : 184
P.pyrifolia         : ------------------------------------------------- :   -
C.sativa            : ------------------------------------------------- :   -
C.papaya            : ------------------------------------------------- :   -
G.max2              : ------------------------------------------------- :   -
H.annuus            : ------------------------------------------------- :   -
L.esculentum1       : ------------------------------------------------- :   -
P.americana         : ------------------------------------------------- :   -
G.max3              : ------------------------------------------------- :   -
S.tuberosum         : ------------------------------------------------- :   -
C.sativus           : ------------------------------------------------- :   -
Z.mays1             : ------------------------------------------------- :   -
Z.mays2             : ------------------------------------------------- :   -
Z.mays4             : ------------------------------------------------- :   -
Z.mays3             : ------------------------------------------------- :   -
S.bicolor           : ------------------------------------------------- :   -
A.thaliana          : ------------------------------------------------- :   -
G.max1              : RSNSLVPYELHEVADAKAEVIDDFAKFNLLLKVKRGQKEEKFKVEVHKNN : 231
L.esculentum2       : RSNSIHPYKLQEIVHANAEMADDSTKLHLVIKTSRGGKEEKFKVQVQHNN : 222
I.batatas           : RSNSLLPYELQEIVHANAEVIDDSAKVHMLIKTKRGEKEEKFSVEVPKNN : 240
O.sativa            : RSNSLFPYELLEIVRAKAEVVEDFAKFDILMKLKRGNKEEKFKAEVHKNL : 231


M.esculentum MecCPI1 : ---------------------- :   -
D.caryophyllus      : ---------------------- :   -
A.vulgaris          : ---------------------- :   -
A.artemisiifolia    : ---------------------- :   -
V.unguiculata       : ---------------------- :   -
R.communis2         : ---------------------- :   -
R.communis1         : EGTFLLNQMEPHT--------- : 209
B.campestris2       : EGVLHLNHMEQQHHD------- : 205
B.campestris1       : EGVLHLNHMEQQHHD------- : 199
P.pyrifolia         : ---------------------- :   -
C.sativa            : ---------------------- :   -
C.papaya            : ---------------------- :   -
G.max2              : ---------------------- :   -
H.annuus            : ---------------------- :   -
L.esculentum1       : ---------------------- :   -
P.americana         : ---------------------- :   -
G.max3              : ---------------------- :   -
S.tuberosum         : ---------------------- :   -
C.sativus           : ---------------------- :   -
Z.mays1             : ---------------------- :   -
Z.mays2             : ---------------------- :   -
Z.mays4             : ---------------------- :   -
Z.mays3             : ---------------------- :   -
S.bicolor           : ---------------------- :   -
A.thaliana          : ---------------------- :   -
G.max1              : QGGFHLNQMEQDHS-------- : 245
L.esculentum2       : EGAFHLNRMEPDN--------- : 235
I.batatas           : EGAFFLNHMAPANS-------- : 254
O.sativa            : EGAFVLNQMQQEHDESSSQ--- : 250
```

Figure 7.2.2.4 Amino acid alignment of 20 plant cystatin sequences. Conserved residues are shown by colour blocking. Blue indicates >80% conservation, cyan indicates > 60% conservation and grey indicates > 40% conservation.

As may be noted in figure 7.2.2.4 a number of sequences show N terminal or C terminal extensions, with similarity to secretory or vacuolar signal sequences respectively. N terminal signal sequences comprise a central region of hydrophobic amino acid residues (A,V, L, I, P, W, F, M) preceded or flanked by regions containing basic amino acids (K, R, H) and serve to direct the nascent polypeptide into the lumen of the endoplasmic reticulum (ER) (Stryer 1988). Subsequently the protein will be directed to the extracellular space (e.g. cell wall or vacuole) unless the specific C-terminal retention signal motif (K/HDEL) is present (Lewin 1996, Nakai 2000). Although prediction of vacuolar localisation is often based on amino acid composition, (Nakai 2000) some authors have proposed that C terminal extensions may serve to direct proteins to the vacuole (Carpin 1999). Protease and protease inhibitor activity has been detected in several cell compartments including peroxisomes, vacuole, golgi body and cell wall (Distephano *et al.* 1999). The cassava MecCPI1 deduced polypeptide, together with several other sequences including cowpea, chestnut, clove pink, ragweed mugwort and papaya sequences contain neither N or C terminal extensions, nor the C terminal peroxisomal targeting motif (S/A/C-K/R/H/-L) and may be cytoplasmic.

The amino acid alignment shown in figure 7.2.2.4 was used to construct an unrooted tree by a distance method (Tajima and Nei algorithm) (Tajima and Nei 1984) within the Treecon package. The resultant dendrogram is shown in figure 7.2.2.5 The observed branching pattern is broadly in agreement with expected phylogenetic relationships, for example monocot and dicot sequences group separately supported by high bootstrap values. Interestingly all the monocot sequences within this group have N terminal extensions, whilst the only other monocot sequence in the analysis (rice) contains a C terminal extension and groups within a dicot C terminal extension containing cluster. The cassava sequence groups with a large dicot cluster of sequences that contain neither N or C terminal extensions.

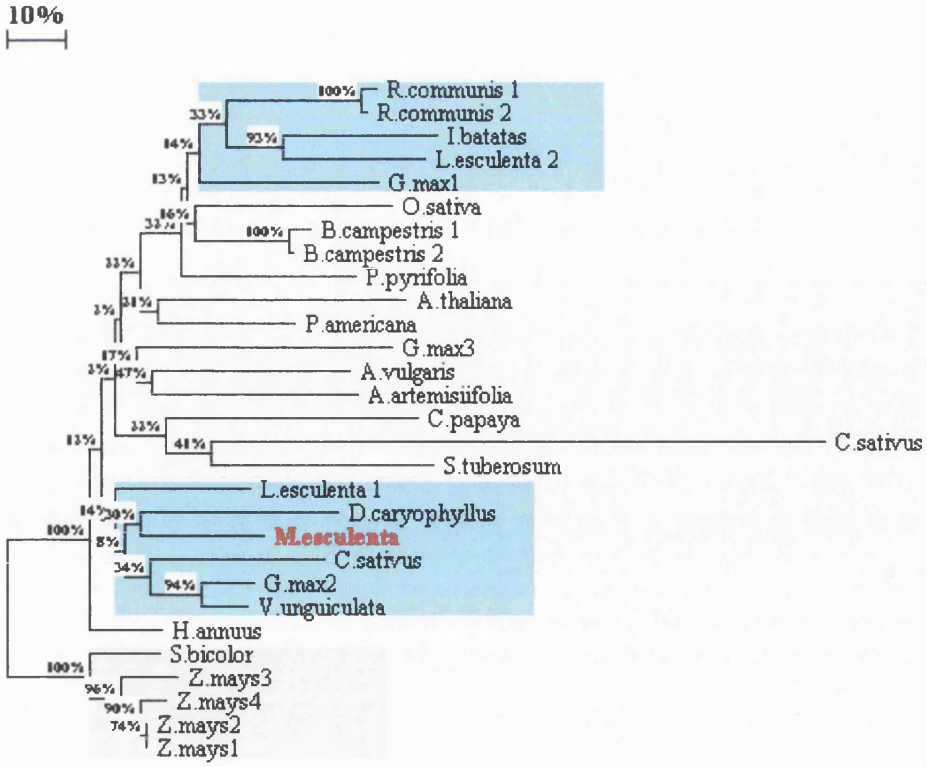


Figure 7.2.2.5 Unrooted tree constructed by a distance method using the ClustalW amino acid alignment shown in figure 7.2.2.3. Bootstrap values (100 replicates) are shown at the nodes. The distance scale indicates percentage of amino acid substitutions per site. A monocot cluster is indicated in grey, groups of sequences which cluster on the basis of N or C terminal extensions are shown in blue.

In order to study the expression of MecCPI1, northern blotting and hybridisation procedures were carried out as described in section 2.7.10 using MecCPI1 as the probe. RNA samples used were prepared from cassava roots stored under field conditions in CIAT, Colombia as previously described in section 7.2.1. Results are shown below in figure 7.2.2.6. The transcript was not detected in freshly harvested root, but was strongly induced by 2 days after harvest.

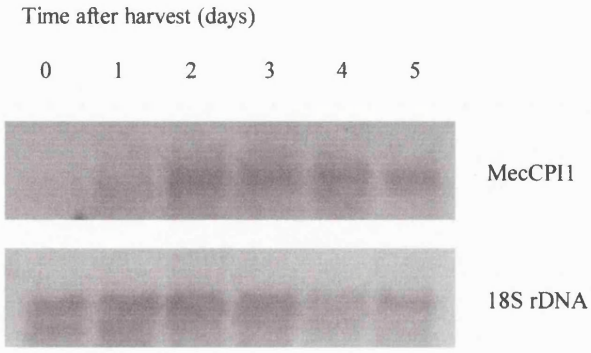


Figure 7.3.2.6 mRNA transcript accumulation of MecCPI1 in cassava storage roots (cultivar MDOM5) during post-harvest physiological deterioration. 10µg total RNA isolated from storage roots at various times after harvest were electrophoresed on a denaturing formaldehyde gel and northern blotted according to standard procedures (Sambrook *et al.* 1989). As a control for equal loading the same blot was stripped and re-hybridised with an 18S rDNA probe (lower panel).

Southern blotting experiments were carried out as described in section 2.7.9. The "Clonemanager" programme was used to generate a restriction map of MecCPI1 and 4 restriction enzymes which did not cut within the cDNA sequence (*Eco*RV, *Xba*I and *Xho*I) or were present as a single restriction site (*Eco*RI) were selected for digestion of genomic DNA. Hybridisation was carried out at 55° C. Data from the high stringency wash (panel B, figure 7.2.2.7) (two 20 minute washes in 0.1X SSC, 0.1% SDS at 60° C) indicate that MecCPI1 occurs in the cassava genome as a single copy gene, containing at least 1 *Xba*I and *Xho*I restriction site which may be located within intron sequences. When compared with the low stringency wash (panel A) (two 30 minute washes in 2X SSC, 0.1% SDS at 55° C), the presence of 2-3 faint bands, particularly in lanes 1, 2 and 3, suggest that a small number of related sequences may also be present in the cassava genome. These data would be consistent with the occurrence of plant cysteine proteases as small gene families (Botella 1996).

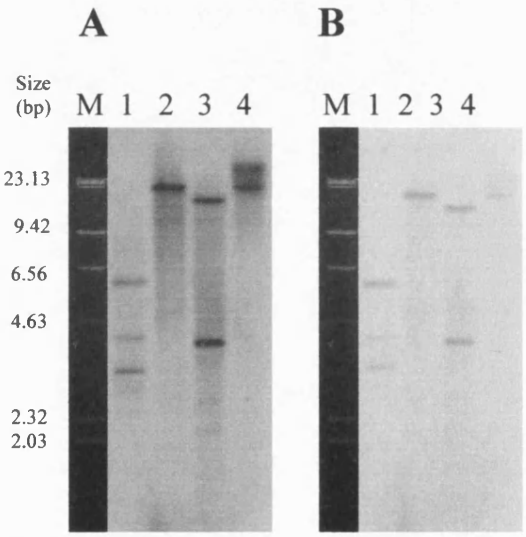


Figure 7.2.2.6 Southern blot analysis of *M. esculenta* nuclear gene organisation. Genomic DNA (20µg per lane) digested with the restriction enzymes *Eco*RI (lane 1), *Eco*RV (lane 2), *Xba*I (lane 3), and *Xho*I (lane 4) and was electrophoresed at 1.5v cm^{-1} on a 0.8% TAE gel and Southern blotted according to standard procedures. The blot was hybridised with MecCPI1 as a probe. Panel A = low stringency wash, panel B = high stringency wash.

7.2.3 Cassava serine protease MecSER

During the course of this project a serine protease of the luecine aminopeptidase class was obtained from the PPD related cDNA library. This clone has been designated MecSER1 (*Manihot esculenta* cDNA encoding Serine protease 1). Insufficient time was available to subclone and sequence the protease, and initial sequence data of the PCR product only is shown in figure 7.2.3.1. The expression profile of the transcript during PPD has not been investigated.

```
rpx7        > TCGAGGATCCGGGTACCATGGGTAGTCTCTTTCGCCTCTTCCTTTCTTCTTTCTTCTTCT
CONSENSUS   > TCGAGGATCCGGGTACCATGGGTAGTCTCTTTCGCCTCTTCCTTTCTTCTTTCTTCTTCT 60

rpx7        > TCTTCTTCTTCTTCTTCCTGTTTTTCACAAAGTTCAGTTCAGCCCCAGTTCTTAGATTCT
CONSENSUS   > TCTTCTTCTTCTTCTTCCTGTTTTTCACAAAGTTCAGTTCAGCCCCAGTTCTTAGATTCT 120

s4          >                                                  AGGAAGCTC.TGGC
rpx7        > CTTTTGCAGTTGCCCCCCTTTGTTATCCTAGAGGAGGAGAGGAGCAAGGAAGCTCATGGC
CONSENSUS   > CTTTTGCAGTTGCCCCCCTTTGTTATCCTAGAGGAGGAGAGGaGCAAGGAAGCTCaTGGC 180

s4          > TCGCTCTTTATCACGTGCC.CTCTCGGCCTTACTCAGCCTGCCAACATCGATGTTCCCAA
rpx7        > TCGCTCTTTATCACGTGCCACTCTCGGCCTTACTCAGCCTGCCAACATCGATGTT.CCAA
CONSENSUS   > TCGCTCTTTATCACGTGCCaCTCTCGGCCTTACTCAGCCTGCCAACATCGATGTTcCCAA 240

s4          > GATCTCTTTTGCTGCAAAAGATGTTGATGTGGTAGAATGGAAAGGAGATATACTTACAGT
rpx7        > GATCTCTTTTGCTGCAAAAGATGTTGATGTGGTAGAATGGAAAGGAGATATACTTACAGT
CONSENSUS   > GATCTCTTTTGCTGCAAAAGATGTTGATGTGGTAGAATGGAAAGGAGATATACTTACAGT 300

s4          > TGGTGTCACTGAGAAAGATATGGCTAAGGATGAAAGCACAAAGTTTCAGAATTCATTTCT
rpx7        > TGGTGTCACTGAGAAAG.TATGGCTAAGGATGAAAGCANAAAGTTTCAGAATTCATTTCT
CONSENSUS   > TGGTGTCACTGAGAAAGaTATGGCTAAGGATGAAAGCAcAAAGTTTCAGAATTCATTTCT 360

s4          > CAAGAAGCTAGATTCTCACTTGGGTGGTCTCTTAAGTGAAGCCTCTTCCGAGGAGGATTT
rpx7        > CAAGANGCNAGNTTCTCACTTGGGTGNTCTCTTAAGT.ANANCTCTTCNAANGAGGANTT
CONSENSUS   > CAAGAaGCtAGaTTCTCACTTGGGTGgTCTCTTAAGTgAarcCTCTTCcrAgGAGGAtTT 420

S5                                                             AAGGGT
ck4         > CACTGGAAAAGCTAGTCAATCCATTGTTCTTAGACTTNCAGGTCTTGGNTCTAAAAGGGT
rpx7        > CAATGG
CONSENSUS   > CAnTGGAAAAGCTAGTCAATCCATTGTTCTTAGACTTnCAGGTCTTGGnTCTAAAAGGGT 480

s5        < TGGCTTAATNGGGCTT.GNCAGTNTGNGTCAACCACNTANCTTTTCGCATTCTGGGTAAG
s4        > TGGCTTAATTGGGCTTGGACAGT
CONSENSUS   > TGGCTTAATtGGGCTTgGaCAGTnTGnGTCAACCACnTAnCTTTTCGCATTCTGGGTAAG 540

s5          < GCAATTGCTGCTATAGCAAAGTCCGCTCAAGCCAGTAATGTTGCTATAGCACTTGCCTCA
CONSENSUS   > GCAATTGCTGCTATAGCAAAGTCCGCTCAAGCCAGTAATGTTGCTATAGCACTTGCCTCA 600

s5          < TCAGAAAGTATCCCAAATGAATCAAAGCTTAATACTGCTTCAGCAATAGCAACTGGAACT
CONSENSUS   > TCAGAAAGTATCCCAAATGAATCAAAGCTTAATACTGCTTCAGCAATAGCAACTGGAACT 660

s5          < GTGCTTGGGATATATGAAGATAACAGGTATAAGTCAGAGTCAAAGAAGCCTGTGCTTAAA
CONSENSUS   > GTGCTTGGGATATATGAAGATAACAGGTATAAGTCAGAGTCAAAGAAGCCTGTGCTTAAA 720

s5          < TCTCTGGATATTCTGGGTCTTGGAATCGGACCTGAAATAGAGAAGAAGCTCAAATATGCT
CONSENSUS   > TCTCTGGATATTCTGGGTCTTGGAATCGGACCTGAAATAGAGAAGAAGCTCAAATATGCT 780

S5          < GGAGATGTTTCTTCTGCTGTAATTTTTGGAAGAGAGCTTGTGAATTCACCAGCAAATGTA
CONSENSUS   > GGAGATGTTTCTTCTGCTGTAATTTTTGGAAGAGAGCTTGTGAATTCACCAGCAAATGTA 840

s5          < CTTACCCCTGCGGTATTGGCGGAAGAAGCTTCGAAGATTGCTTCCGCACATAGCGATGTT
CONSENSUS   > CTTACCCCTGCGGTATTGGCGGAAGAAGCTTCGAAGATTGCTTCCGCACATAGCGATGTT 900

rpx7r       <                          GCAGAGCAATGCAAAGAGTTAAAAATGGGGTCTTATCTG
s5          < ATTTCTGCTACCATCTTGAATGCAGAGCAATGCAAAGAATTAAAAATGGGGTCTTATCTG
CONSENSUS   > ATTTCTGCTACCATCTTGAATGCAGAGCAATGCAAAGArTTAAAAATGGGGTCTTATCTG 960

rpx7r       < GGTNTTGCTGCAGCTTCTGCAAATCGCACCTCATTTCANCCATTTGTGTTATAAGCCTCC
s5          < GGTGTTGCTGCAGCTTCTGCAAATCGCACCTCATT
CONSENSUS   > GGTgTTGCTGCAGCTTCTGCAAATCgCACCTCATTTCAnCCATTTGTGTTATAAGCCTCC 1020

rpx7r       < AAGTGGACCTGTTAAAGCCCCATGGTACCCGGATCCTCGATTCTTTTGNTTTT
CONSENSUS   > AAGTGGACCTGTTAAAGCCCCATGGTACCCGGATCCTCGATTCTTTTGnTTTT  1073
```

Figure 7.2.3.1 Consensus output of gcg GELASSEMBE for PCR product MecSER1. λ vector sequence either side of the insert is shown in bold. The insert is 1016 bp in size and contains two internal *Eco*RI sites shown in red.

7.3 Characterization of cDNA clones involved in transcription and translation

7.3.1 Cassava translation initiation factor eIF-5A

The sequence and deduced translation of a cassava translation initiation factor with homology to plant, mammalian and yeast eIF-5A is shown in figure 7.3.1.2. The clone was designated MecTIF (*Manihot esculenta* cDNA encoding Translation Initiation Factor eIF-5A) and has been submitted to the Genbank database under the accession number AF266464. The clone is 925 bp in size and encodes a predicted protein of 160 amino acids. The sequencing strategy used is shown below (figure 7.3.1.1)

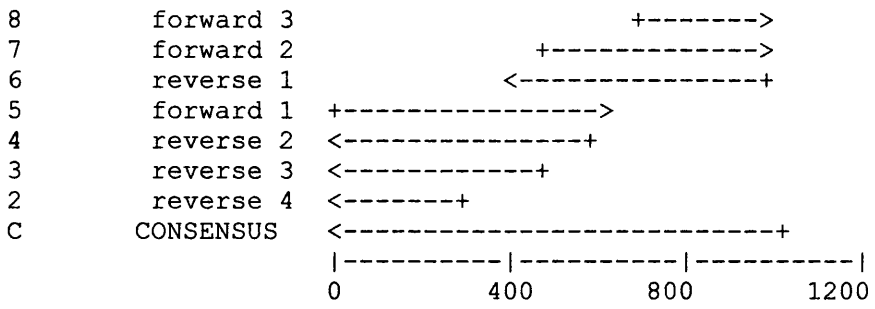


Figure 7.3.1.1 GELASSEMBLE output showing sequencing strategy for MecTIF. Universal M13/pUC primers were used for initial forward and reverse sequencing reactions. For subsequent internal sequencing reactions oligonucleotide primers were designed based on sequence data obtained, using the "Primer Designer" software programme.

The nucleotide sequence contains a short 5' UTR with the start codon located in the sequence context ACGCTATGT, similar to the Kozak consensus CCGCCATGG identified as optimal for eukaryotic translation initiation. The majority of plant and vertebrate genes contain purines (A or G) at the –3 and +4 positions relative to the start ATG codon and this is believed to be important for fidelity of translation initiation (Kozak 1986, Joshi 1997). Interestingly, the cassava eIF5-A sequence, in common with the other plant and yeast sequences, contains the pyrimidine T at the +4 position allowing the formation of a conserved Ser residue as the second amino acid. The animal sequences in contrast contain G in the +4 position giving Ala as the second amino acid. The ORF terminates with an "ochre" (TAA) termination signal at nucleotide 531, with a second adjacent "opal" (TGA) termination signal located at 537. The 3' UTR contains an exact polyadenylation signal (consensus AATAAA) although it is located 94 bp upstream of the poly(A) addition site.

```
1     caagagacaaattgaattgaaaacaaaccctagaagaaagaaaaaacgctatgtcggacg
                                                        M  S  D
61    aggaacaccacttcgagtccaaggccgatgctggagcctccaagacctttccacaacaag
        E  E  H  H  F  E  S  K  A  D  A  G  A  S  K  T  F  P  Q  Q
121   ctggtaccattcgcaagaatggctacatcgtaatcaagaatcgtccctgcaaggttatgg
        A  G  T  I  R  K  N  G  Y  I  V  I  K  N  R  P  C  K  V  M
181   aggtttcaacatcaaagacgggaaaacatggtcatgctaagtgccattttgttggaattg
        E  V  S  T  S  K  T  G  K  H  G  H  A  K  C  H  F  V  G  I
241   atatattcaatggaaaaaaacttgaagatattgttccttcatcccacaattgtgatgttc
        D  I  F  N  G  K  K  L  E  D  I  V  P  S  S  H  N  C  D  V
301   ctcatgttaaccgtactgattatcagctgattgatatctctgaagatggttttgtgagtc
        P  H  V  N  R  T  D  Y  Q  L  I  D  I  S  E  D  G  F  V  S
361   ttctgactgaaactggaaacaccaaggacgatctcaggcttcccaccgatgaaaatctgc
        L  L  T  E  T  G  N  T  K  D  D  L  R  L  P  T  D  E  N  L
421   tcagccagattaaagatgggtttgctgaggggaaggacctcgtagtgagcgtcatgtctg
        L  S  Q  I  K  D  G  F  A  E  G  K  D  L  V  V  S  V  M  S
481   caatgggagaggagcggatatgttcccttaaggacattggtcctaaaaattaaagatgat
        A  M  G  E  E  R  I  C  S  L  K  D  I  G  P  K  N
541   gctatagcattagctgcagctgatggtgaaacagtctctatgtttgctgacaaggaattt
601   tttatctactgcgcatatgtacaacaatcttctattaagacagtattttagctgtgtaat
661   gaagacctttaaggcttgtatggaggaggtgcggaaaggaactgaagctgctcatttcct
721   gatggagggaactcgggtgatttggaaggatatggaatatataaatatttctgttttata
781   tatttcctggttattggagataataaaggtatgtatcacctccctgaggacacatgttt
841   ccccgttaaatttctgataatgagagctttaaaagccaactaatgtttgtcattttaaaa
901   aaaaaaaaaaaaaaaaaaaaaaaaaaaa
```

Figure 7.3.1.2 Nucleotide sequence and deduced translation of the cassava translation initiation factor eIF5-A (cDNA clone MecTIF). Nucleotide sequence features – the start ATG codon, predicted stop codons, and the putative polyadenylation signal are shown in red. Within the deduced polypeptide sequence a putative glycosylation site is shown in blue. The conserved hypusination motif is shown highlighted and in blue, whilst the hypusinated lysine (K) residue is highlighted in pink.

Computer analysis of the deduced protein indicates a molecular weight of 17.62 kDa and a predicted isoelectric point of 5.8. These values are within the expected range for eIF5-A proteins (Mw 16-18 and pI 5-6) (Pay *et al.* 1991). Analysis with PSORT (www.psort.nibb.ac.jp) and related programmes indicate that carboxy or amino terminal targeting sequences are not present and that the protein is probably cytoplasmic (P= 0.65). Indeed a cytoplasmic location would be expected for a eukaryotic translation factor, and in mammalian studies eIF5-A is ubiquitously cytoplasmic (Shi *et al.* 1996). An amino acid alignment of the predicted cassava eIF5-A with all other available plant sequences as well as a yeast and animal sequences is shown in figure 7.3.1.3. Sequences used were *Zea mays* (maize), *Oryzae sativa* (rice), *Euphorbia esula* (leafy spurge), *Medicago sativa* (alfalfa), *Nicotinia plumbaginifolia* (curled leaf tobacco), *Nicotinia tabacum* (common tobacco), *Solanum tuberosum* (potato), *Arabidopsis thaliana* (thale cress), *Senecio vernalis* (senecio), *Saccharomyces cerevisiae* (yeast), *Spodoptera exigua* (beet armyworm), and *H.sapiens* (human). The alignment shows the high degree of conservation of eIF5-A even across kingdoms, with the sequence of 12 amino acids

surrounding the K → hypusine post-translational modification site showing highest conservation. As noted by Dresselhaus *et al.* (1999) this region forms a major basic cluster of amino acids as shown in figure 7.3.1.4, and forms the functional domain of the protein. In the analysis of Dresselhaus *et al.* this basic cluster was highly conserved amongst eukaryotes and showed 70% identity with the corresponding region in a non-eukaryote Archaea sequence. The core of the conserved consensus hypusination motif is GKHGxAK, the first K residue is modified to hypusine. As noted by Pay *et al.* (1991) the *S.cerevisiae* sequence, although more similar to the animal sequences in the central and carboxy terminal parts of the protein, is highly similar to the plant sequences at the amino terminus and dissimilar to the animal sequences. The cassava clone was highly similar to other plant eIF5-A sequences with 89% overall identity to the Euphorb *E.esula* (Leafy spurge) and with 51% similarity to the human (*H.sapiens*) sequence.

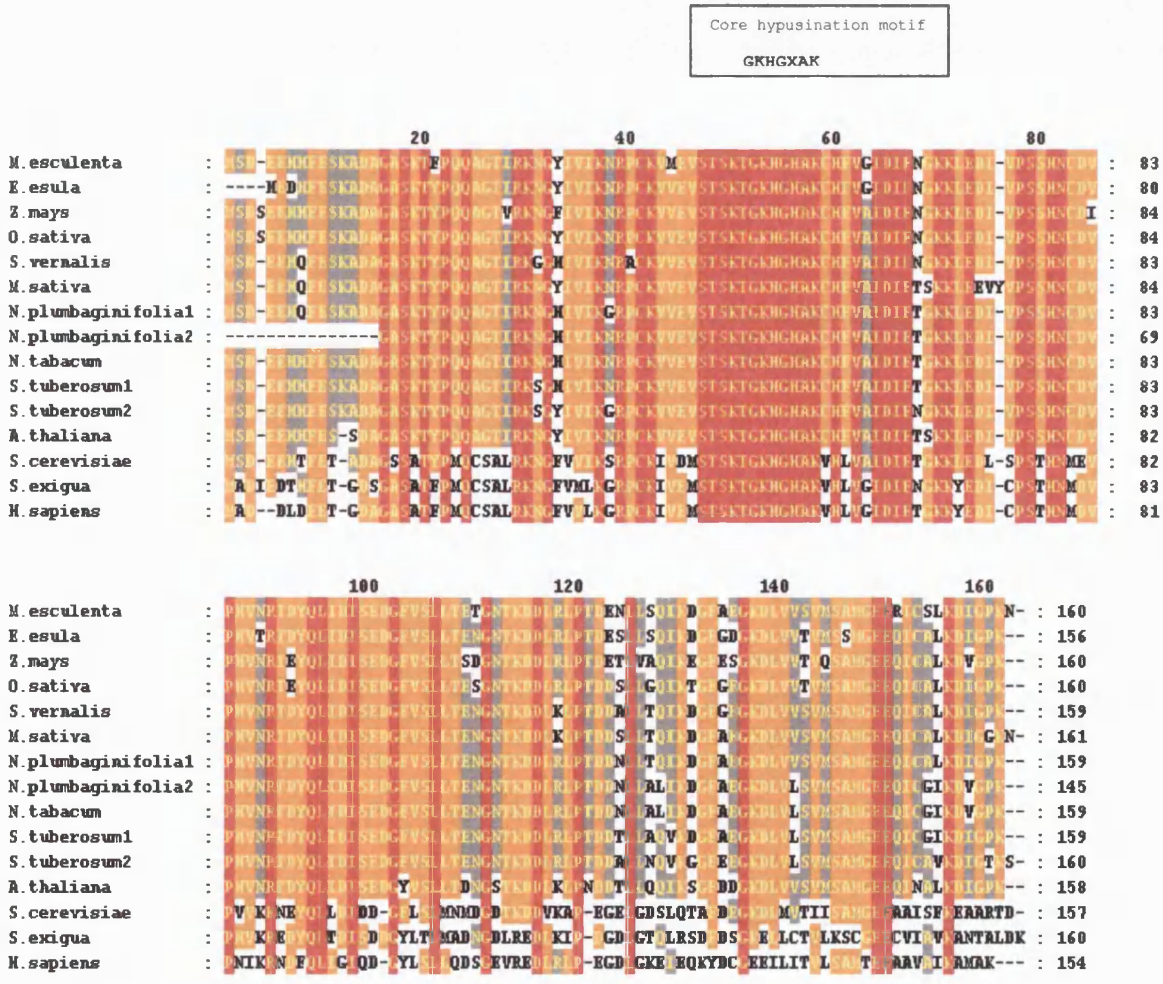


Figure 7.3.1.3 Amino acid alignment of plant, yeast and animal eIF5-A sequences. Conserved residues are shown in colour blocking. Red indicates 100% conservation, orange indicates > 80% conservation and grey indicates > 60% conservation. The core of the hypusination motif is indicated in text.

Figure 7.3.1.4 Partial amino acid alignment of plant, yeast and animal eIF5-A sequences. Conserved physiochemical properties of amino acid residues are shown in colour blocking. Green blocks indicate basic amino acids, blue blocks indicate negatively charged amino acids, blue text indicates aliphatic amino acids and pink text indicates hydrophobic residues.

The amino acid alignment shown in figure 7.3.1.3 was used to construct an unrooted tree by a distance method (Tajima and Nei algorithm) within the Treecon package. The resultant dendrogram is shown in figure 7.3.1.5. The observed branching pattern is broadly in agreement with expected phylogenetic relationships and with previously published eIF5-A trees (Dresselhaus *et al.* 1999, Gerloff *et al.* 1998). The plant and animal sequences form discrete groups supported by high bootstrap values, with the *S.cerevisiae* sequence grouping separately between these, again supported by high bootstrap values. Within the plant group a large Solanaceae cluster comprising *Nicotinia* and *Solanum* species is observed, as is a smaller Euphorbiaceae cluster containing the *M.esculenta* and *E.esula* sequences. The *Arabidopsis* sequence groups separately as the only representative of the Brassicaceae. Interestingly a mixed cluster containing the *S.vernalis* (Asteraceae), *M.sativa* (Fabaceae) and the second *N.plumbaginifolia* (Solanaceae) sequence is present. This grouping of the 3 sequences is also present in the tree of Dresselhaus *et al.* (1999). The bootstrap values for this branching pattern are not high and since relatively few plant eIF5-A sequences are available it is likely that addition of further sequences from the respective groups will clarify this ambiguity. A second puzzle was that although the monocot *Z.mays* sequence grouped separately to the dicot sequences as would be expected, the *O.sativa* sequence grouped within the dicots, albeit with a bootstrap value of less than 25%. Again, addition of further plant sequences to the analysis may clarify the situation. As might be expected from evidence of differential expression of eIF5-A isoforms in tobacco and potato (Chamot and Kuhlemier 1991, Fugino and Kituka 1997) the tree

also shows evidence of sorting on a functional as well as phylogenetic basis. The second *N.plumbaginifolia* sequence groups outside the major Solanaceae cluster whilst the 2 *S.tubersum* sequences also group separately indicating that during the course of evolution different genetic isoforms of eIF5-A may have become specialised for different physiological roles.

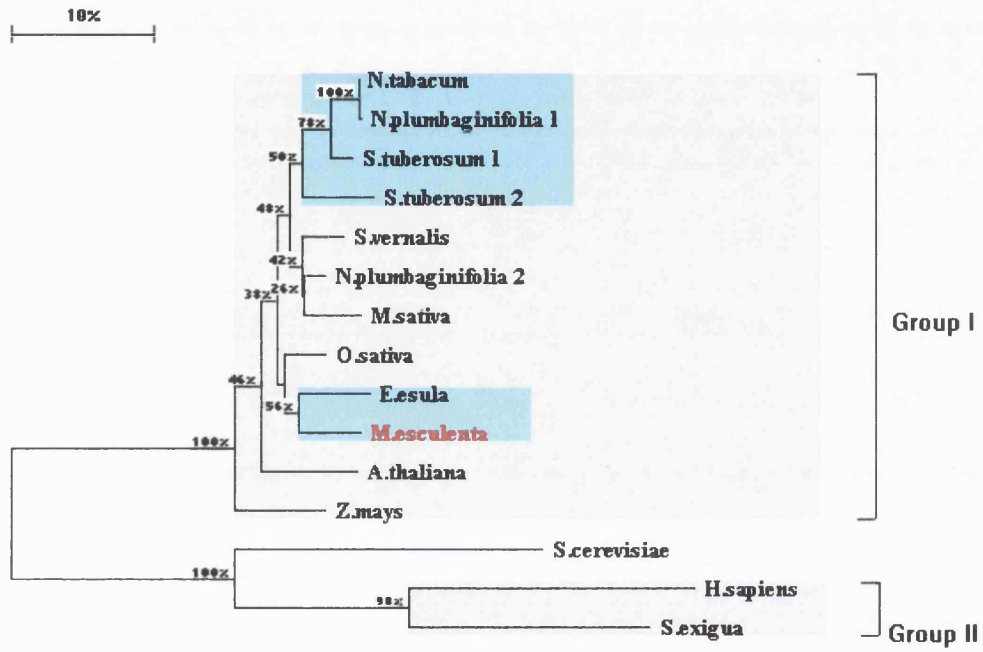


Figure 7.4.1.5. Unrooted tree constructed by a distance method using the ClustalW amino acid alignment shown in figure 7.4.1.3. Bootstrap values (100 replicates) are shown at the nodes. The distance scale indicates percentage of amino acid substitutions per site. Solanaceae and Euphorbiaceae clusters are indicated in blue, plant and animal groups are indicated in grey.

Little has been published regarding the structure, gene organisation and expression of plant eIF-5A genes. Since the cloning of the first example in 1991 in alfafa (*Medicago sativa*) (Pay *et al.* 1991) a dozen or so sequences have been published, but with little further analysis. To date 2 eIF5-A sequences have been isolated from *Nicotinia plumbaginifolia* and 5 from *Solanum tuberosum*, although only 2 of these have been published (Dresselhaus *et al.* 1999). In *Z.mays* a single sequence has been isolated, however Southern blotting indicates the presence of at least 3 eIF5-A genes. These data indicate that plant eIF-5A sequences occur as small gene families. To investigate eIF5-A gene organisation in cassava, Southern blotting experiments were carried out as

described in section 2.7.9. The "Clonemanager" (Scientific and Educational software, version 4) programme was used to generate a restriction map of MecTIF and 2 restriction enzymes which did not cut within the cDNA sequence – *Bgl*II and *Eco*RI – were selected for digestion of genomic DNA. Hybridisation was carried out overnight at 55° C using the MecTIF insert as probe. For the low stringency wash to allow detection of related sequences the following regime was used – two 30 minute washes in 2X SSC. 0.1% SDS at 55° C, followed by two 20 minute washes in 1X SSC, 0.1% SDS at 60° C. Following autoradiography, the membrane was re-washed at high stringency (two 20 minute washes in 0.1X SSC, 0.1% SDS) to allow detection of the cognate gene only. Results are shown below (figure 7.4.1.6). Four hybridising bands are present in the *Eco*RI digest lane following low stringency washing, whilst 5 or 6 bands are evident in the *Bgl*II lane. After the high stringency was 2 bands remain in the *Bgl*II lane, whilst a single hybridising band is seen in the *EcoRI* lane. These data suggest that the cassava eIF5-A clone described here occurs in the genome as a single copy gene and that a *Bgl*II restriction site but not an *Eco*RI restriction site is present within intron sequences of this gene. Data from the low stringency wash suggests MecTIF is a member of a small gene family comprising a small number of cassava eIF5-A genes.

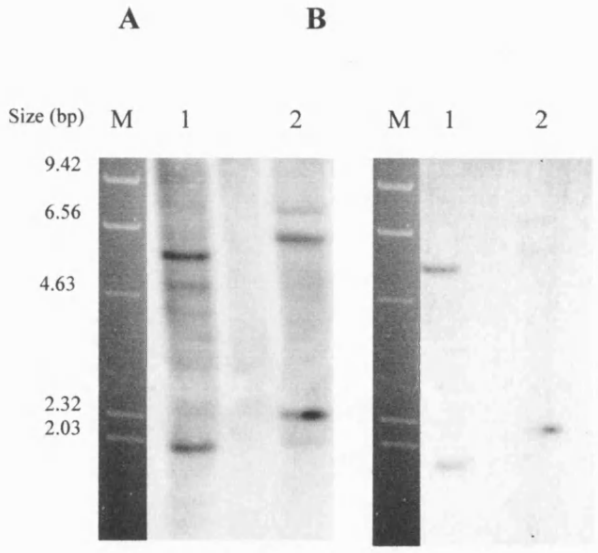


Figure 7.3.1.6 Southern blot analysis of *M. esculenta* nuclear gene organisation. Genomic DNA (20μg per lane) digested with the restriction enzymes *Bgl*II (lane 1), or *Eco*RI (lane 2), was electrophoresed at 1.5v cm^{-1} on a 0.8% TAE gel and Southern blotted according to standard procedures. The blot was hybridised with MecTIF as a probe. Panel A = low stringency wash, panel B = high stringency wash .

7.3.2 Cassava RNA polymerase subunit RPB8

The sequence and deduced translation of a cassava RNA polymerase subunit with homology to yeast RPB8 is shown in figure 7.3.2.2 The clone was designated MecRPB8 (*Manihot esculenta* cDNA encoding RPB8) and has been submitted to the Genbank database under the accession number AF266463.

As discussed in section 7.2.2 the sequence was originally isolated as a chimeric clone designated MecCPI/RNApol. The sequence and deduced translation of this chimeric clone is shown in appendix C. A PCR based strategy was employed in order to subclone the RPB8 sequence as described in section 7.2.2. The new clone in plasmid pGEM T-easy containing RPB8 sequences only was designated MecRPB8 and was fully sequenced as shown in figure 7.3.2.1 A "Blast 2 sequences" search was carried out using the NCBI software (http://www.ncbi.nlm.nih.gov) and showed 100% identity between MecRPB8 and the relevant part of clone MecCPI/RNApol, indicating that no mismatch errors had been introduced during the PCR process.

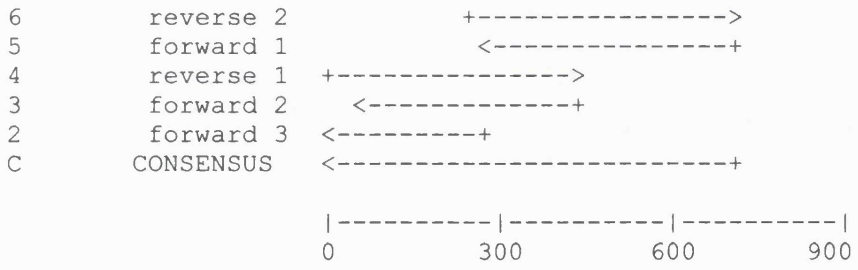


Figure 7.3.2.1 GELASSEMBLE output showing sequencing strategy for MecRPB8. Universal pUC/M13 primers were used for initial forward and reverse sequencing reactions. For subsequent internal sequencing reactions oligonucleotide primers were designed based on sequence data obtained, using the "Primer Designer" software programme.

The nucleotide sequence is 617 bp in length and encodes a predicted protein of 110 residues. The ORF terminates with an "opal" (TGA) stop codon. No sequences approaching the consensus polyadenylation signal (AATAAA) are found in the 3' UTR.

```
1     gtgacatgttcatgcatttagatgtgaacacagaaatatatccaatggctgtaggtgata
         D  M  F  M  H  L  D  V  N  T  E  I  Y  P  M  A  V  G  D
61    aattcactatggcattggcacacactctaaatttagatggaacacctgacactgggtatt
       K  F  T  M  A  L  A  H  T  L  N  L  D  G  T  P  D  T  G  Y
121   acattcagggagcgaggaagacccttgcagacaaatatgaatacataatgcatggaaagc
       Y  I  Q  G  A  R  K  T  L  A  D  K  Y  E  Y  I  M  H  G  K
181   tatacaagatctcagaggaaggttcaggaaaagcagttaaagcggagatatatgtttcat
       L  Y  K  I  S  E  E  G  S  G  K  A  V  K  A  E  I  Y  V  S
241   atggtggacttctaatgatgctgaaaggagatccttctcatgtctctcacttcgaacttg
       Y  G  G  L  L  M  M  L  K  G  D  P  S  H  V  S  H  F  E  L
301   accagcggctatttcttcttataaggaagttgtgaagcccttgttctaatttgattcttt
       D  Q  R  L  F  L  L  I  R  K  L  -
361   agctgttgttcctgatgccatcttattaagggcaaaattattgccttgattgagattttc
421   tcacttgtcttcagtgtctaaactgaatttgaacttggaaaacttaaactatgtggtgta
481   tttgaatcttatgtggctaatcaggcatgtaacttaagtttaaaaaaaaaaaaaaaaaa
541   aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
601   aaaaaaaaaaaaaaaa
```

Figure 7.3.2.2 Nucleotide sequence and deduced translation of the cassava RNA polymerase subunit RPB8 clone MecRPB8. The internal primer used for amplification of the RPB8 sequence is shown in blue. The predicted stop codon is shown in red. The aspartic acid (D) residue referred to in the text is shown in grey.

Computer analysis of the deduced protein indicates a molecular weight of 12.5 kDa and a predicted isoelectric point of 6.2. These values are slightly lower and higher respectively than those cited for other RPB8 sequences (14-16 kDa and pI 4.3) (Woychik *et al.* 1990, McKune *et al.* 1995, Sakurai and Ishihama 1997) and combined with the amino acid alignment with other RPB8 sequences (figure 7.3.2.3) suggest the cassava sequence is truncated at the 5' end. The aspartic acid (D) residue preceding the first methionine (M) of the ORF is thus included as it forms a conserved polar amino acid found in all other sequences analysed. The sequence was found to have no highly significant PROSITE motifs, and other motif searches only suggested that the sequence was indeed a member of the RPB8 family. As noted by Woychik *et al.* (1990) the absence of recognisable sequence motifs for DNA and nucleoside triphosphate binding indicate the subunit does not have a catalytic role but may be important for a function shared by all 3 RNA polymerases - such as transcriptional efficiency, nuclear localisation or co-ordinate regulation of tRNa, mRNa and rRNA synthesis. Since RPB8 would be translated in the cytoplasm but functions in the nucleus the presence of a

nuclear localisation signal (NLS) might be expected. Such NLS sequences do not consist of a conserved sequence motif, but comprise a cluster of 3 or more basic lysine (K) and arginine (R) residues commonly preceded by a proline (P) residue (Lewin 1997). Motifs similar to an NLS are present in the amino terminal part of the other RPB8 sequences (between residues 20 to 35), however this region is absent in the truncated cassava sequence presented here. An amino acid alignment of cassava MecRPB8 with the only other available plant sequence as well as yeast and animal sequences is shown in figure 7.3.2.3. Sequences used were *Arabidopsis thaliana* (cress), *Saccharomyces cerevisiae* (yeast), *Schizosaccharomyces pombe* (yeast), *Pedinomonas minutissima* (marine phytoplankton), *Caenorhabdis elegans* (nematode), and *H.sapiens* (human). Several regions of the predicted protein showed complete conservation across kingdoms. The cassava sequence showed 61% amino acid identity to the *Arabidopsis* sequence, with lowest identity (34%) with the human RPB8 sequence.

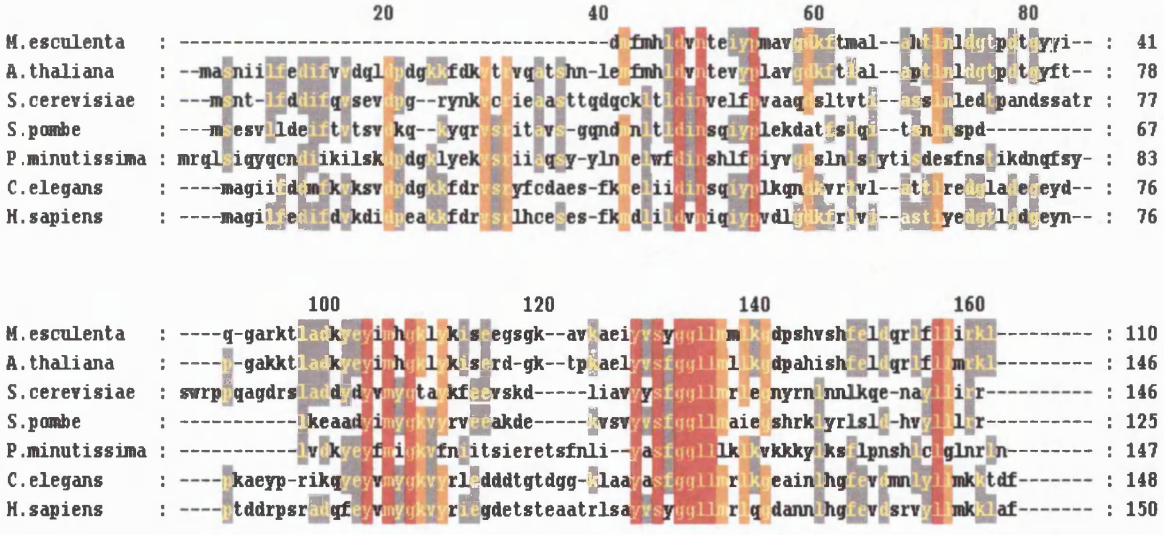


Figure 7.3.2.3 Amino acid alignment of plant, yeast and animal RPB8 sequences. Conserved residues are shown in colour blocking. Red indicates 100% conservation, orange indicates > 80% conservation and grey indicates > 60% conservation.

To investigate RPB8 gene organisation in cassava, Southern blotting experiments were carried out as described in section 2.7.9. The "Clonemanager" programme was used to generate a restriction map of MecRPB8 and 4 restriction enzymes which did not cut within the cDNA sequence – *Eco*RI, *Eco*RV, *Xba*I, and *Xho*I – were selected for digestion of genomic DNA. Hybridisation was carried out overnight at 55° C using the

MecRPB8 insert as probe. The final wash stringency for the low stringency wash was 1X SSC, 0.1% SDS at 60° C. Following autoradiography, the membrane was re-washed at high stringency (two 20 minute washes in 0.1X SSC, 0.1% SDS) to allow detection of the cognate gene only. Results are shown below (figure 7.3.2.4). These data indicate that RPB8 is encoded by a single copy gene in cassava, as is the case in *S.cereviasiae* (Woychik *et al.* 1990), with a small number of related sequences also present.

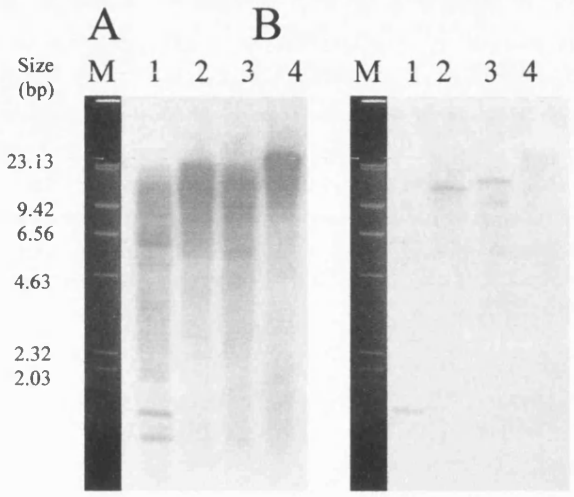


Figure 7.3.2.4 Southern blot analysis of *M. esculenta* nuclear gene organisation. Genomic DNA (20μg per lane) digested with the restriction enzymes *Eco*RI (lane 1), *Eco*RV (lane 2), *Xba*I (lane 3) and *Xho*I (lane 4) was electrophoresed at 1.5v cm^{-1} on a 0.8% TAE gel and Southern blotted according to standard procedures. The blot was hybridised with MecRPB8 as a probe. Panel A = low stringency wash, panel B = high stringency wash

7.4 Conclusions and discussion

The RNA polymerase subunit MecRPB8 and translation initiation factor MecTIF, although of theoretical interest, were not thought likely to play a role in cassava root PPD. For this reason northern analysis was not carried out and these clones will not be discussed further.

The isolation of the proteases and cystatin clone, and the up regulation of MeCASP1 and MecCPI1 during the deterioration response raise the intriguing possibility that cassava PPD may include an active senescence process. However, aside from the work of Lalaguna and Agudo (1989) and Huang *et al.* (2000), no further research has been carried out and it remains unclear whether PPD of the cassava storage root should more properly be considered as an active PCD programme triggered by wounding and/or

oxidative stress caused by wounding; or whether it reflects physiological changes resulting from wounding which are accompanied by necrotic (non programmed) cell death. Certainly, from a selective viewpoint, it would be advantageous for the cassava plant to institute a rapid, programmed, breakdown of a detached non-propagative storage root which could act as a source of pathogenic organisms.

Approaches which have been successfully used to extend models of animal PCD to plant systems include detection of DNA ladders reflecting ordered DNA fragmentation by gel electrophoresis (Wang *et al.* 1996, Schmit *et al.* 1999); or *in situ* labelling of fragmenting DNA by the TUNEL assay (Wang *et al.* 1996). The mammalian PARP assay using heterologous bovine poly(ADP-ribose) has been used to detect PARP cleavage – a characteristic of apoptotic animal cells – by plant extracts (D'Silva *et al.* 98).

If PPD of the cassava storage root did show characteristics of PCD, further studies on the role of MecASP1 transcript up-regulation during PPD would be of interest.

Strategies which could be used include extension of northern analysis of different tissue types and immunohistochemical or *in situ* hybridisation approaches in order to determine if the transcript shows tissue specific localisation and/or stage specific expression. Protein gel electrophoresis approaches could also be used to determine if aspartic proteases show altered processing during PPD.

Since serine proteases have also been implicated in PCD, northern analysis to determine the expression profile of this transcript during PPD would be of interest.

The role of up-regulation of MecCPI1 is unclear. Does it reflect a wound induced defence protein active against pests (inhibition of exogenous cysteine proteases), or could it play a role in modulation of PCD (inhibition of endogenous cysteine proteases)? Recently a number a cassava cysteine protease clones have been isolated in this laboratory (Tzimas, unpublished results) and it would be of interest to determine if these are substrates of MecCPI1. Approaches which have generally been described in the literature to study the function of cystatins have involved expression of the inhibitor in *E.coli* and comparison of its inhibitory effect on cysteine proteases from different sources (for example see Doi-Kawano *et al.* 1998, Pernas *et al.* 1998). A similar approach could be used in cassava, for example, inhibitory effects on cysteine protease activity in root extracts could be compared with inhibitory effects on those from insect extracts and commercial cysteine proteases of different types.

If such experiments did indicate that cassava PPD was or involved a component of a PCD/ senescence response this could allow perhaps a more direct route to control of PPD via either exogenous treatment with cytokinins or transgenic approaches. For example in an elegant study described by Gan and Amasino (1996) an isopentenyl transferase gene was fused to a leaf senescence specific promoter forming an auto-regulatory construct. Initiation of leaf senescence in the trangenic tobacco plants induced the promoter, resulting in increased cytokinin levels. Increased cytokinin levels in turn inhibited senescence and turned off the promoter.

CHAPTER EIGHT:
GENERAL DISCUSSION

8.1 Summary of results and general conclusions

Results presented here indicate the occurrence of a transient wound induced oxidative burst in the cassava storage root, which could participate as an initial wound signal within the root. Superoxide (O_2^-) was produced with 15 minutes after injury and had declined by 7-10 hours after injury. H_2O_2 was detected within 3 hours after injury and showed a peak in accumulation at 24-27 after injury.

Of the reactive oxygen scavenging enzymes, superoxide dismutase showed little change at either the protein or transcriptional level whilst catalase and peroxidase were both up regulated during the post harvest storage period. These data would suggest that superoxide dismutase does not play a significant role in the development of PPD. In other plant systems, an extracellular superoxide dismutase is believed to catalyse the conversion of O_2^- to H_2O_2 during the oxidative burst (Scheel 1998). Since no significant differences in H_2O_2 accumulation were observed between susceptible and less susceptible cultivars, this would again suggest that susceptible and less susceptible cultivars experience similar initial oxidative stress, in terms of H_2O_2 accumulation, but may respond slightly differently downstream of the initial production of H_2O_2. Differences in catalase levels were observed at both the protein and transcriptional level, with levels of MecCAT1 transcript and overall catalase activity being more pronounced in less susceptible cultivars. In addition, roots from pruned plants, which are less susceptible to PPD, showed higher levels of MecCAT1 transcript accumulation than roots from non pruned controls. In contrast, overall peroxidase activity and MecPX1 transcript accumulation was lower in less susceptible cultivars and the transcript was not up-regulated in response to pre-harvest pruning. Taken together these data suggest that roots from less susceptible cultivars or from pruned plants may efficiently utilise catalase to scavenge H_2O_2 produced after wounding, resulting in the formation of molecular oxygen and water:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

In more susceptible cultivars where peroxidase levels are relatively higher, and catalase levels relatively lower, a higher proportion of H_2O_2 scavenging would occur via peroxidase mediated reactions requiring the participation of cellular components as an electron donor:

$$H_2O_2 + 2AH \rightarrow 2H_2O + 2A$$

With regard to potential signalling components, MecCAT1 was up-regulated in response to ethylene treatment and pre-harvest pruning, and showed slight down regulation in response to methyl jasmonate treatment. MecPX1 showed strong up-regulation in response to ethylene treatment but was unaffected by pre-harvest pruning or methyl jasmonate. The superoxide dismutase probe showed slight up-regulation in response to pre-harvest pruning, but was unaffected by ethylene or methyl jasmonate. Since both catalase and peroxidase levels appear to play a role in modulating the PPD response, these results would support a role for ethylene in modulating PPD. Previous studies in cassava have indicated production of ethylene in the cassava storage root initiating around 6 hours after injury and continuing to increase over a 22 hour period (Plumbley *et al.* 1981, Hirose *et al.* 1984a). Results presented here suggest that ethylene produced in response to injury could serve to drive expression of MecCAT1 and MecPX1. The up-regulation of catalase and superoxide dismutase transcripts, but not MecPX1 in response to pre-harvest pruning suggests that pre-wounding or pre-stressing of the plant is capable of transmitting some sort of systemic signal to the roots that alters the gene expression profile within the root. The slight down regulation of MecCAT1 in response to methyl jasmonate treatment, and the lack of effect of methyl jasmonate on the peroxidase and superoxide dismutase transcripts was somewhat surprising, since jasmonic acid and its methyl ester, methyl jasmonate, are involved as intracellular signal molecules which mediate gene activation in response to wounding in other plant systems. One could hypothesise that down regulation of catalase by methyl jasmonate would have the effect of allowing increased production of H_2O_2, which has also been proposed as a second messenger during defence signal transduction. Since both catalase and peroxidase are ROS scavengers, MecCAT1 and MecPX1 may be directly or indirectly up-regulated by H_2O_2 and/or by ethylene as discussed above. In any event, the observed up-regulation of these PPD related genes does not appear to be mediated via the octadecanoid/lipoxygenase pathway.

Localisation data presented here for peroxidase and H_2O_2 accumulation, provide considerable support for the hypothesis of Tanaka *et al.* (1983) and Wheatley and Schwabe (1985), particularly when coupled with the fluorescence microscopy observations of Buschmann *et al.* (2000c). These authors have proposed that the that the symptoms of blue/black vascular streaking observed as PPD result from a peroxidase mediated oxidation of one of the fluorescent coumarin compounds, probably scopoletin,

that show transient accumulation in the cassava storage root after injury. Localisation experiments presented here, show that in the early stages after wounding both peroxidase activity and H_2O_2 accumulation is predominantly observed the cells surrounding the xylem vessels. The fluorescent microscopy studies of Buschmann *et al.* (2000c) have indicated that the fluorescent coumarin components scopolin, scopoletin and esculin first begin to accumulate in the area surrounding the xylem vessels within 6-12 hours after injury. Thus, all of the components required for peroxidase mediated oxidation of scopoletin would be present in such cells within 24 hours after injury.

With regard to an overall view of the processes occurring during PPD, a schematic model is presented in figure 8.1 that aims to draw together results obtained during this study with previous analyses. It is clear that many of the processes occurring in the cassava root after injury are similar to those of wound and defence responses in other plant systems. The formation of tyloses and occlusions in the xylem vessels, and localised accumulation of fluorescent coumarin compounds has been described in other plant systems in response to wounding and pathogen attack, and has been described in cassava leaves infected with *Xanthamonas campestris* (Kpemoua *et al.* 1996). Although the rapid production O_2^- and H_2O_2 reported here could function as an initial wound stress signal, other signals could operate in addition. In other plants, phloem mobile signals such as systemin, hydraulic signals and electrical signals have been proposed as the initial wound signal (Schaller and Ryan 1995, Malone 1996). Pre-harvest pruning of the cassava plant has been shown to cause increased toughness and lignification of the root. This increased toughness could function to reduce the susceptibility of the roots at least partially by decreasing the amount of damage during harvest, thereby reducing the initial wound signal. The isolation of proteases and protease inhibitor cDNAs which have been implicated in programmed cell death, and the up-regulation of MecASP1 and MecCPI1 within 24 and 48 hours after injury respectively, raises the intriguing possibility that PPD of cassava storage roots may include components of programmed cell death as is seen in other plant defence responses such as the HR. Indeed, several parallels could be drawn between PPD and the HR. Both processes are active ones and can be inhibited by cyloheximide; increases in ethylene production have been noted before the onset of HR cell death; as have accumulation of fluorescent coumarin components including scopoletin, up-regulation of PAL and β-glucanases; carotenoid degradation; membrane breakdown and

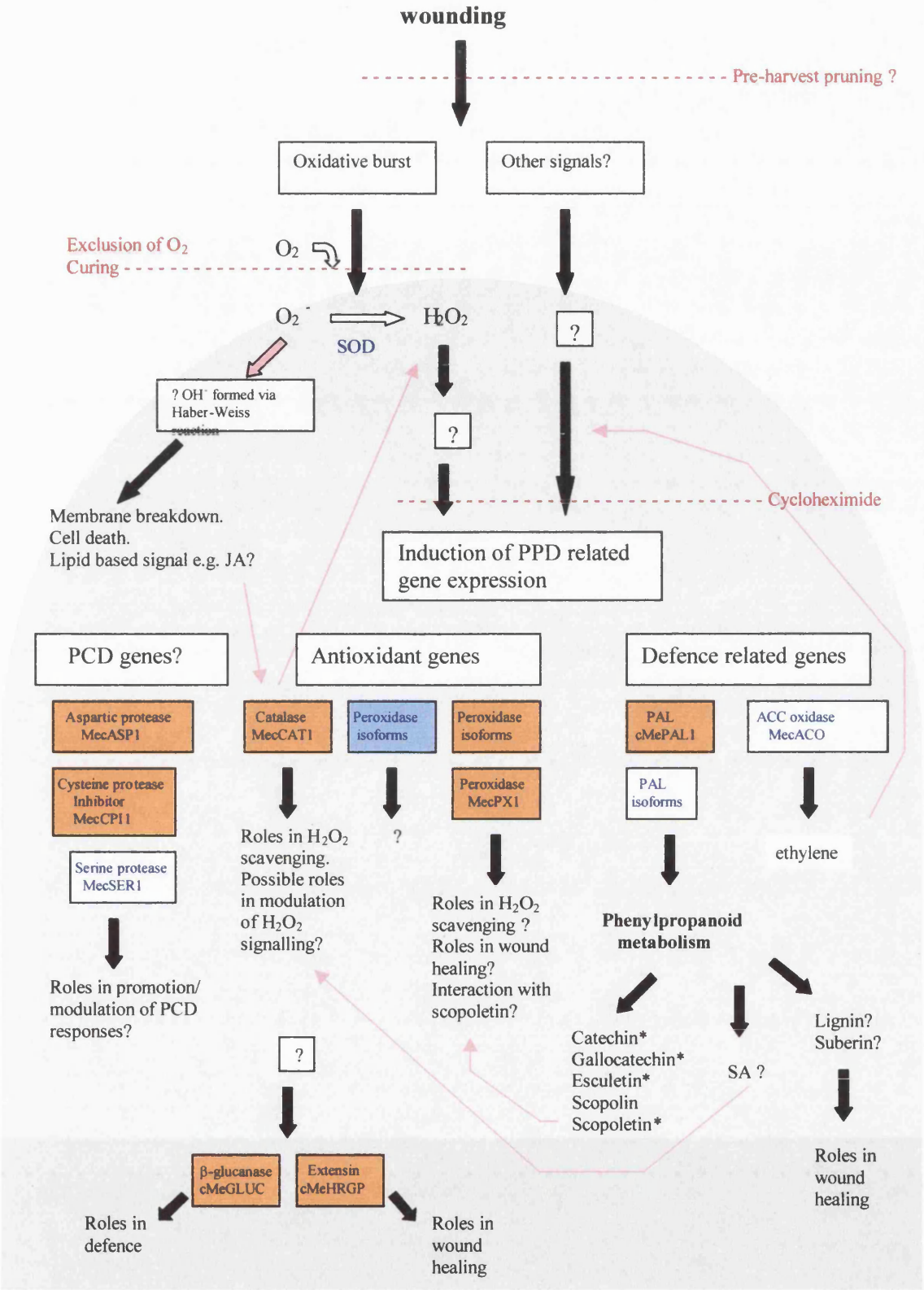


Figure 8.1 Schematic model representing processes that may be occurring during post-harvest physiological deterioration of the cassava storage root. Genes or proteins known to be expressed during PPD are indicated in blue text. Those known to be up-regulated are indicated in orange boxes, those known to be down regulated are indicated in blue boxes. Possible interactions between components in the model are indicated by pink arrows. Unknown components are indicated with a question mark. Possible points where treatments known to inhibit PPD may act are indicated in red. Of the secondary phenolic compounds known to accumulate in the root after injury, those which are capable of acting as antioxidants are marked with an asterisk. Genes which are expressed in the later stages after injury (after 48 hours) are indicated towards the base of the figure on a dark grey background.

increases in peroxidase and catalase activity (Goodman *et al.* 1986, Scheel 1998, Pontier *et al.* 1999). In leaves undergoing the HR, catalase levels have been reported to drop in the area of tissue undergoing hypersensitive cell collapse, but to remain high in surrounding tissue (Milosovic and Slusarenko 1996) which could be considered as analogous to differences in susceptible and less susceptible roots. In this same study no significant differences were noted in superoxide dismutase activity or isoform pattern, whilst peroxidase activity increased several-fold in the area of tissue undergoing hypersensitive cell death.

Cell death certainly does occur during PPD of the cassava storage root, however no studies have been carried out to determine if such cell death reflects necrotic cell death or a form of programmed cell death. It has been proposed by several authors that programmed cell death is a default pathway in both plants and animals, and it may be that severance of the root from the plant and associated wounding results in the triggering of such a pathway. Although cassava storage roots can produce a wound periderm under conditions of high temperature and humidity albeit slower than other root crops, under normal storage conditions this does not occur. It is likely that the failure of wound healing and sealing has the effect of driving gene expression away from re-establishment of homeostasis and towards continued defence responses. Experiments which have not been carried out here, but would be of interest in elucidating the mechanisms behind PPD could include extension of northern analysis of the clones presented here and elsewhere (Han 2000, Li unpublished results) to include H_2O_2 and cytokinin treatment of roots. Exogenous application of cytokinin has been demonstrated to inhibit senescence responses and the formation of HR lesions in other plants (Pontier *et al.* 1999) and it would of interest to determine if cytokinin application could inhibit PPD. Further study on the type of cell death occurring during PPD and on the differences in root gene expression patterns between pruned and non pruned plants would also be of interest.

BIBLIOGRAPHY

Abler M and Scandalios J (1993) Isolation and characterisation of a genomic sequence encoding the maize *Cat3* gene. Plant Molecular Biology **22**: 1031-1038

Abrahams S, Hayes C and Watson J (1996) Organ specific expression of three peroxidase encoding cDNAs from Lucerne. Australian Journal of Plant Physiology **23**: 551-555

Adam A, Bestwick C, Barna B, and Mansfield J (1995) Enzymes regulating the accumulation of active oxygen species during the hypersensitive reaction of bean to *Pseudomonas syringae* pv. *phaseolicola.* Planta **197**: 240-249

Adewusi S and Bradbury J (1993) Carotenoids in cassava - comparison of open-column and HPLC methods of analysis. Journal of the Science of Food and Agriculture **62**: 375-383

Alami I, Jouy N and Clerivet A (1999) The lipoxygenase pathway is involved in elicitor induced phytoalexin accumulation in plane tree cell suspension cultures. Journal of Phytopathology **147**: 515-519

Alarcon J and Malone M (1995) The influence of plant age on wound induction of proteinase inhibitors in tomato. Physiologia Plantarum **95**: 423-427

Allan A and Fluhr R (1997) Two distinct sources of elicited active oxygen species in tobacco epidermal cells. The Plant Cell **9**: 1559-1572

Allem A, Mendes A, Salomao A and Burle M (2000a) The value of aggregation of Manihot genetic resources. *In* Carvalho L, Thro A, and Vilarinhos A (eds) Cassava Biotechnology. Proceedings of the 4th International Scientific Meeting, Salvador, Brazil 1998, Brasilia 2000 pp15-23

Allem A, Roa A, Mendes A, Salomao A, Burle M, Second G, Carvalho P and Cavalcanti J (2000b) The primary gene pool of cassava. *In* Carvalho L, Thro A, and Vilarinhos A (eds) Cassava Biotechnology. Proceedings of the 4th International Scientific Meeting, Salvador, Brazil 1998, Brasilia 2000 pp 3-14

Alvarez M and Lamb C (1997) Oxidative burst-mediated defense responses in plant disease resistance. *In* Scandalios J (ed) Oxidative Stress and the Molecular Biology of Antioxidant Defenses. CSHL Press, Cold Spring Harbor pp 815-839

Alvarez M, Pennell R, Meijer P, Ishikawa A, Dixon R and Lamb (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. Cell **92**: 773-784

Amiot M, Fleuriet A, Cheynier V, Nicolas J (1997) Phenolic compounds and oxidative methods in fruit and vegetables. *In* Tomas-Barberan A, Robins R (eds) Phytochemistry of Fruit and Vegetables: Proceedings of the Phytochemical Society of Europe 41 Oxford University Press pp51-85

Anderson M, Chen Z and Klessig D (1998) Possible involvement of lipid peroxidation in salicylic acid mediated induction of PR-1 gene expression. Phytochemistry **47**: 555-566

Ardi R, Kobiler I, Jacoby B, Keen N and Prusky D (1998) Involvement of epicatechin biosynthesis in the activation of the mechanism of resistance of avocado fruits to *Colletotrichum gloeosporoides.* Physiological and Molecular Plant Pathology **53**: 269-285

Arias-Garzon D and Sayre R (2000) Genetic engineering approaches to reducing cyanide toxicity in cassava. *In* Carvalho L, Thro A, and Vilarinhos A (eds) Cassava Biotechnology. Proceedings of the 4th International Scientific Meeting, Salvador, Brazil 1998, Brasilia 2000 pp 213-221

Asada, K (1992) Ascorbate peroxidase - a hydrogen peroxide-scavenging enzyme in plants. Physiologia Plantarum **85**: 235-241

Auh C and Murphy T (1995) Plasma-membrane redox enzyme is involved in the synthesis of O_2- and H_2O_2 by *Phytophthora* elicitor-stimulated rose cells. Plant Physiology **107**: 1241-1247

Averre C (1967) Vascular streaking of stored cassava roots. Proceedings of the First International Symposium on Tropical Root Crops, Trinidad **2**: IV31-IV35

Bach M, Schnitzler J, and Seitz H (1993) Elicitor induced changes in Ca 2+ influx, K+ efflux and 4-hydroxybenzoic acid synthesis in protoplasts of *Daucus carota.* Plant Physiology **103**: 407-412

Baker C and Orlandi E (1995) Active oxygen in plant pathogenesis. Annual Reviews of Phytopathology **33**: 299-321

Baron C, Zambryski P (1995) The plant response in pathogenesis, symbiosis and wounding: variations on a common theme? Annual Review of Genetics **29**: 107-129

Bartosz G (1997) Oxidative stress in plants. Acta Physiologiae Plantarum **19**: 47-64

Beeching J, Dodge A, Moore K, Phillips H and Wenham J (1994) Physiological deterioration in cassava: possibilities for control. Tropical Science **34**: 335-343

Beeching J, Dodge A, Moore, K , and Wenham, J (1995) Physiological deterioration in cassava: An incomplete wound response? *In* Thro A and Roca W (eds.) The Cassava Biotechnology Network: Proceedings of the Second International Scientific Meeting. 1995. Cali, Colombia pp 729-736

Beeching J, Han Y and Cooper R (1997) Physiological deterioration in cassava: towards a molecular understanding. African Journal of Root and Tuber Crops **2**: 99-105

Beeching J, Han Y, Gomez-Vasquez R, Day R, Cooper RM (1998) Wound and defence responses in cassava as related to post-harvest physiological deterioration. *In* Romeo J, Downum K, Verpoorte R (eds.) Phytochemical Signals and Plant-Microbe Interactions, Plenum Press, New York pp 231-249

Bergey D, Orozco-Cardenas M, de Moura D and Ryan C (1999) A wound and systemin inducible polygalacturonase in tomato leaves. Proceedings of the National Academy of Sciences of the USA **96**: 1756-1760

Bowler C, Van Camp W, Van Montagu M and Inze D (1994) Superoxide dismutase in plants. Critical Reviews in Plant Sciences **13**: 199-218

Bravo J, Fita I, Gouet P, Jouve H, Melik-Adamyan W and Murshudov G (1997) Structure of catalases. *In* Scandalios J (ed) Oxidative Stress and the Molecular Biology of Antioxidant Defenses CSHL Press, Cold Spring Harbor pp 407-445

Bradley D, Kjellbom P and Lamb C (1992) Elicitor-induced and wound-induced oxidative cross-linking of a proline-rich plant-cell wall protein - a novel, rapid defense response. Cell **70**: 21-30

Brownleader M, Ahmed N, Trevan M, Chaplin M and Dey P (1995) Purification and partial characterisation of tomato extensin peroxidase. Plant Physiology **109**: 1115-1123

Brownleader M, McNally P, Davies G, Trevan M and Dey P (1997) Elicitor-induced extensin insolubilization in suspension-cultured tomato cells. Phytochemistry **46**: 1-9

Bjellqvist, B, Basse B, Olsen E and Celis J (1994) Reference points for comparisons of two-dimensional maps of proteins from different human cell types defined in a pH scale where isoelectric points correlate with polypeptide compositions. Electrophoresis **15**: 529-539

Bode W, Engh R, Musil D, Thiele U, Huber R, Karshikov A, Brzin J, Kos J, and Turk V (1988) The 2.0 A X - ray crystal structure of chicken white cystatin and its possible mode of interaction with cysteine proteases. EMBO Journal. **7**: 2593-2599

Boeuf G, Bauw G, Legrand B and Rambour S (2000) Purification and characterization of a basic peroxidase from the medium of cell suspension cultures of chicory. Plant Physiology and Biochemistry **38**: 217-224

Bolwell P, Butt V, Davies D and Zimmerlin A (1995) The origin of the oxidative burst in plants. Free Radical Research **23**: 517-532

Bolwell (1996) The origin of the oxidative burst in plants. Biochemical Society Transactions **24**: 438-442

Bolwell G and Wojtaszek P (1997) Mechanisms for the generation of reactive oxygen species in plant defence - a broad perspective. Physiological and Molecular Plant Pathology **51**: 347-366

Bolwell P, Blee K, Butt V, Davies D, Gardner S, Gerrish C, Minibayeva F, Rowntree E, and Wojtaszek P (1999) Recent advances in understanding the origin of the apoplastic oxidative burst in plant cells. Free Radical Research **31**: 137-145

Bolwell (1995) The origin of the oxidative burst in plants. Biochemical Society Transactions **24**: 438-442

Booth R (1974) Post harvest deterioration of tropical root crops. Tropical Science **16**: 49-63

Booth R (1976) Storage of fresh cassava (*Manihot esculenta*). I. Post-harvest deterioration and its control. Experimental Agriculture **12**: 103-111.

Botella M, Xu Y, Prabha T, Zhao Y, Narisimhan M, Wilson K, Nielson S, Bressan R and Hasegawa P (1996) Differential expression of soybean cysteine proteinase inhibitor genes during development and in response to wounding and methyl jasmonate. Plant Physiology **112**: 1201-1210

Bowler C, Alliotte T, De Loose M, Van Montagu M and Inze D (1989) The induction of manganese superoxide-dismutase in response to stress in *Nicotiana-plumbaginifolia.* EMBO Journal **8**: 31-38

Bowler C, Van Camp W, Van Montagu M and Inze D (1994) Superoxide dismutase in plants. Critical Reviews in Plant Science **13**: 199-218

Bravo J, Fita I, Gouet P, Jouve H, Melik-Adamyan W, Murshudov G (1997) Structure of catalases. *In* Scandalios J (ed) Oxidative Stress and the Molecular Biology of Antioxidant Defences, Cold Spring Harbor Laboratory Press, Cold Spring Harbor pp 407-446

Brendel V, Bucher P, Nourbabakhsh I, Blaisdel BE, Karlin S (1992) Methods and algorithms for statistical analysis of protein sequences. Proceedings of the National Academy of Sciences of the USA **89**: 2002-2006

Buchannan-Wollaston V and Ainsworth C (1997) Leaf senescence in *Brassica napus*: cloning of senescence related genes by subtractive hybridisation. Plant Molecular Biology **33**: 821-834

Buchannan-Wollaston V (1997) The molecular biology of leaf senescence. Journal of Experimental Botany **48**: 181-199

Burle M, Allem A, Abadie T, Costa I, Fakuda W (2000) The use of environmental maps in GIS as a tool for cassava genetic resource classification. *In* Carvalho L, Thro A, and Vilarinhos A (eds) Cassava Biotechnology. Proceedings of the 4th International Scientific Meeting, Salvador, Brazil 1998, Brasilia 2000 pp 24-33

Buschmann H, Rodriguez M, Tohme J, Beeching J (2000a) Qualitative and quantitative changes of phenolic components of cassava roots during post-harvest physiological deterioration. *In* Carvalho L, Thro A, and Vilarinhos A (eds.) Cassava Biotechnology. Proceedings of the 4th International Scientific Meeting, Salvador, Brazil 1998, Brasilia 2000 pp 517-525

Buschmann H, Reilly K, Rodriguez M, Tohme J and Beeching J (2000b) Hydrogen peroxide and flavan-3-ols in root tubers of cassava during post harvest physiological deterioration. Journal of Agricultural and Food Chemistry **48**: 5522-5529

Buschmann H, Rodriguez M, Tohme J and Beeching J (2000c) Accumulation of hydroxycoumarins during post-harvest deterioration of tuberous roots of cassava. Annals of Botany **86**: 1153-1160

Cabral G, Carvalho L and Schaal B (2000) The formation of the storage root in cassava. *In* Carvalho L, Thro A, and Vilarinhos A (eds.) Cassava Biotechnology. Proceedings of the 4th International Scientific Meeting, Salvador, Brazil 1998, Brasilia 2000 pp 354-356

Campa A (1991) Biological roles of plant peroxidases: known and potential functions. *In* Everse J, Everse K and Grisham J (eds) Peroxidases in Chemistry and Biology vol 2. CRC press, Boca Raton pp 25-50

Campos A and de Carvalho V (1990) Deterioracao pos-colheita de mandioca. Pesquisa Agropecuaria Brasileira **25**: 773-781

Carpin S, Crevecoeur M, Greppin H and Penel C (1999) Molecular cloning and tissue specific expression of an anionic peroxidase in zucchini. Plant Physiology **120**: 799-810

Catesson A (1992) Plant peroxidases and cell differentiation: cyto- and histological aspects. *In* Penel C, Gaspar T and Greppin H (eds.) Plant Peroxidases 1980-1990, Topics and Detailed Literature on Molecular, Biochemical and Physiological Aspects. University of Geneva

Cazale A, Rouet-Mayer M, Barbier-Brygoo H, Mathieu Y and Lauriere C (1998) Oxidative burst and hypo-osmotic stress in tobacco cell suspensions. Plant Physiology **116**: 659-669

Chamot D and Kuhlemeier C (1992) Differential expression of genes encoding the hypusinate containing translation initiation factor, eIF-5A, in tobacco. Nucleic Acids Research **20**: 665-669

Chance B, Boveris A, Oshino N and Loschen G (1973) The nature of catalase intermediate and its biological function. *In* King TE (ed.) Oxidases and Related Redox Systems, University Park Press, Baltimore pp 350-353

Chapple I (1997) Reactive oxygen species and antioxidants in inflammatory disease. Journal of Clinical Periodontology **24**: 287-296

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Reporter **11**: 113-116

Cheigh H, Um S and Lee C (1995) Antioxidant characteristics of melanin related products from enzymatic browning reaction of catechin in a model system. *In* Chang L and Whitaker J (eds.) Enzymatic Browning and its Prevention pp 200-208

Chen F and Foolad M (1997) Molecular organisation of a gene in barley which encodes a protein similar to aspartic protease and its specific expression in nucellar cells during degeneration. Plant Molecular Biology **35**: 821-831

Chen Z, Silva H, Klessig D (1993) Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science **262**: 1883-1886

Chong J, Baltz R, Fritig B and Saindrenan P (1999) An early salicylic acid-, pathogen- and elicitor-inducible tobacco glucosyltransferase: role in compartmentalization of phenolics and H_2O_2 metabolism. FEBS Letters **458**: 204-208

Cock JH (1985) Cassava: New Potential for a Neglected Crop. Westfield Press, Boulder.

Cohen S, Chang A, and Hsu L (1972) Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichiae coli* by R factor DNA. Proceedings of the National Academy of Sciences of the USA **69**: 2110-2114

Cohen G (2000) Protein-protein interactions in the initiation of caspase activation in apoptosis. Biochemist **22**: 25-27

Collen J and Pedersen M (1994) A stress-induced oxidative burst in *Eucheuma platycladum* (Rhodophyta). Physiologia Plantarum **92**: 417-422

Cortes DF, Reilly K, Beeching J, Iglesias C and Tohme J (2000) Mapping wound response genes involved in post harvest physiological deterioration (PPD) of cassava. *In press.*

Crevcoeur M, Pinedo M, Greppin H and Penel C (1997) Peroxidase activity in shoot apical meristems from *Spinacia.* Acta Histochemisty **99**: 177-186

Criqui M, Jamet E, Parmentier Y, Marbach J, Durr A, and Fleck J (1992) Isolation and characterization of a plant cDNA showing homology to animal glutathione peroxidases. Plant Molecular Biology **18**: 623-827

Croft K, Voisey C and Slusarenko A (1990) Mechanism of hypersensitive cell collapse: correlation of increased lipoxygenase activity with membrane damage in leaves of *Phaseolus vulgaris* inoculated with an avirulent race of *Pseudemonas syringae.* Physiological and Molecular Plant Pathology **36**: 49-62

Czyhrinciw N and Jaffe W (1951) Modificaciones quimicas durante la conservacion de raices y tuberculos. Archivos Venezolanos de Nuticion **2**: 49-67

D'Auzac J (1996) Toxic oxygen: protection against pathogens. Plantations, Recherché, Developpement Mai-Juin 1996: 164-170

D'Hondt K, Stack S, Gutteridge S, Vandekerckhove J, Krebbers E and Gal S (1997) Aspartic protease genes in the Brassicaceae *Arabidopsis thaliana* and *Brassica napus.* Plant Molecular Biology **33**: 187-192

D'Silva I, Poirier G and Heath M (1998) Activation of cysteine proteases in cowpea plants during the hypersensitive response – a form of programmed cell death. Experimental Cell Research **245**: 389-399

Dangl J (1998) Plants just say NO to pathogens. Nature **394**: 525-527

Dat J, Lopez-Delgado H, Foyer C, Scott I (1998) Parallel changes in hydrogen peroxide and catalase during thermotolerance induced by salicylic acid or heat acclimation in mustard seedlings. Plant Physiology **116**: 1351-1357

Data E, Quevedo M and Gloria L (1984) Pruning techniques affecting the root quality of cassava at harvest and subsequent storage. *In* Uritani I and Reyes (eds.) Tropical Root Crops: Postharvest Physiology and Processing, JSSP, Tokyo pp 127-143

Deiss LP, Galinka H, Berissi H, Cohen O and Kimchi A (1996) Cathepsin D protease mediates programmed cell death induced by interferon gamma, Fas/Apo-1 and TNF alpha. EMBO Journal **15**: 3861-3870

Del Rio L, Pastori G, Palma J, Sandalio L, Sevilla F, Corpas F, Jiminez A, Lopez-Huertas E and Hernandez J (1998) The activated oxygen role of peroxisomes in senescence. Plant Physiology **116**: 1195-1200

Dellaporta S, Wood J, and Hicks J (1983) A plant DNA minipreparation. Plant Molecular Biology Reporter **1**: 19-21

Desikan R, Hancock J, Coffey M and Neill S (1996) Generation of active oxygen in elicited cells of *Arabidopsis thaliana* is mediated by a NADPH oxidase like enzyme. FEBS Letters **382**: 213-217

Desikan R, Reynolds A, Hancock J, and Neill S (1998) Harpin and hydrogen peroxide both initiate cell death but have differential effects on defence gene expression in *Arabidopsis* suspension cultures. Biochemical Journal **330**: 115-120

Desikan R, Clarke A, Atherfold P, Hancock J and Neill S (1999) Harpin induces mitogen activated protein kinase activity during defence responses in *Arabidopsis thaliana* suspension cultures. Planta **210**: 97-103

Devereux J, Haeberli P, and Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Research **12**: 387-95

Distephano S, Palma J, Gomez M and del Rio L (1997) Characterisation of endopeptidases from plant peroxisomes. Biochemical Journal **327**: 399-405

Distephano S, Palma J, McCarthy I, and del Rio L (1999) Proteolytic cleavage of plant proteins by peroxisomal endoproteases from senescent pea leaves. Planta **209**: 308-313

Dixon R, Harrison M and Lamb C (1994) Early events in the activation of plant defence responses. Annual Review of Phytopathology **32**: 479-501

Doke N (1983a) Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. Physiological Plant Pathology **23**: 345-357

Doke N (1983b) Generation of superoxide anion by potato tuber protoplasts upon the hypersensitive response to hyphal wall components of *Phytophthora infestans* and specific inhibition of the reaction by a suppressor of hypersensitivity. Physiological Plant Pathology **23**: 359-367

Doke N (1985) NADPH dependant O_2^- generation in membrane fractions isolated from wounded potato tubers inoculated with *Phytophthora infestans*. Physiological Plant Pathology **27**: 311-322

Doke N, Miura Y, Sanchez L, Park H, Noritake T, Yoshioka H and Kawakita K (1996) The oxidative burst protects plants against pathogen attack: mechanism and role as an emergency signal for plant bio-defence – a review. Gene **179**: 45-51

Doke N (1997) The oxidative burst: roles in signal transduction and plant stress. *In* Scandalios JG (ed) Oxidative Stress and the Molecular Biology of Antioxidant Defences. Cold Spring Harbor Laboratory press, Cold Spring Harbor pp 785-813

Doi-Kawano K, Kouzuma Y, Yamasaki N and Kimura M (1998) Molecular cloning, functional expression and mutagenesis of a cDNA encoding a cysteine proteinase inhibitor from sunflower seeds. Journal of Biochemistry **124**: 911-916

Dong X (1998) SA, JA, ethylene, and disease resistance in plants. Current Opinion in Plant Biology **1**: 316-323

Dorey S, Kopp M, Geoffroy P, Fritig B and Kauffmann S (1999) Hydrogen peroxide from the oxidative burst is neither necessary nor sufficient for hypersensitive cell death induction, phenylalanine ammonia lyase stimulation, salicylic acid accumulation, or scopoletin consumption in cultured tobacco cells treated with elicitin. Plant Physiology **121**: 163-171

Dornenburg H and Davies C (1999) The relationship between lipid oxidation and antioxidant content in post harvest vegetables. Food Reviews International **15**: 435-453

Dresselhaus T, Cordts S, and Lorz, H. (1999) A transcript encoding translation initiation factor eIF-5A is stored in unfertilised egg cells of maize. Plant Molecular Biology **39**: 1063-1071

Edwards R, Stones SM, Gutierrezmellado MC and Jorrin J (1997) Characterization and inducibility of a scopoletin-degrading enzyme from sunflower. Phytochemistry **45**: 1109-1114

Esaka M, Maeshima M and Asahi T (1983) Mechanism of the increase in catalase activity through microbody development in wounded sweet potato root tissue. Plant and Cell Physiology **24**: 615-623

Esaka M, Yamada N, Kitabayashi M, Setoguchi Y, Tsugeki R, Kondo M, and Nishimura M (1997) cDNA cloning and differential gene expression of three catalases in pumpkin. Plant Molecular Biology **33**: 141-155

Eshdat Y, Holland D, Faltin Z and Benhayyim G (1997) Plant glutathione peroxidases. Physiologia Plantarum **100**: 234-240

Essers S (2000) Working group 7- cyanogenesis. Convener/reporter Sandra Essers. *In* Carvalho L, Thro A, and Vilarinhos A (eds) Cassava Biotechnology. Proceedings of the 4th International Scientific Meeting, Salvador, Brazil 1998, Brasilia 2000 pp 622-624

Fakuda W (1998) Farmer participation in cassava biotechnology research. Revista Brasileira de Mandioca **17**: 79

FAO Plant Production and Protection Paper 130 (1995) Post-harvest deterioration in cassava: a biotechnology perspective. Wenham J (ed), FAO, Rome

FAO FAOSTAT Database (1998) http://apps.fao.org/

FAO (2000) The world cassava economy. FAO and IFAD, Rome 2000

Faro C, Ramalho-Santos M, Vieira M, Mendes A, Simoes I, Andrade R, Verissimo P, Lin X, Tang J and Pires E (1999) Cloning and characterization of cDNA encoding cardosin A, and RGD-containing plant aspartic proteinase. Journal of Biological Chemistry **274**: 28724-28729

Felsenstein J (1994) PHYLIP version 3.5 User Manual. University of Washington, Seattle

Foyer C, Lopez-Delgado H, Dat J and Scott I (1997) Hydrogen peroxide and glutathione associated mechanisms of acclimatory stress tolerance and signaling. Physiologia Plantarum **100**: 241-254

Fregene M, Angel F, Gomez R, Rodriguez F, Chavarriaga P, Roca W, Tohme J and Bonierbale M (1997) A molecular genetic map of cassava (*Manihot esculenta* crantz). Theoretical and Applied Genetics **95**: 431-441

Fregene M, Okogbenin E, Angel F, Suarez M, Guitierez J, Chavarriaga P, Roca W, Tohme J and Bonierbale M (2000) Genome mapping in cassava improvement: challenges, achievements and opportunities. *In* Carvalho L, Thro A, and Vilarinhos A (eds) Cassava Biotechnology. Proceedings of the 4th International Scientific Meeting, Salvador, Brazil 1998, Brasilia 2000 pp 192-201

Frison E (1994) Sanitation techniques for cassava. Tropical Science **34**: 146-153

Frugoli J, Zhong H, Nuccio M, McCourt P, McPeek M, Thomas T and McClung C (1996) Catalase is encoded by a multigene family in *Arabidopsis thaliana* (L Heynh). Plant Physiology **112**: 327 -336

Frugoli J, McPeek M, Thomas T and McClung C (1998) Intron loss and gain during evolution of the catalase gene family in angiosperms. Genetics **149**: 355-365

Fugino J and Kituka Y (1997) Nucleotide sequence of 5 cDNAs encoding eukaryotic translation initiation factor 5A (eIF5-A) from potato. Plant Physiology **115**: 864

Gan S and Amasino R (1997) Making sense of senescence. Plant Physiology **113**: 313-319

Garcia-Martinez J and Moreno J (1986) Proteolysis of ribulose 1,5-biphosphate carboxylase/oxygenase in citrus leaf extracts. Physiologia Plantarum **66**: 377-383

Geloff D, Joachimiak M, Cohen F, Cannarozzi G, Chamberlain S, and Benner S (1998) Structure prediction in a post genomic environment: a secondary and tertiary structural model for the initiation factor 5a family. Biochemical and Biophysical Research Communications **251**: 173-181

Giesemann A, Biehl B and Lieberei R (1986) Identification of scopoletin as a phytoalexin of the rubber tree *Hevea brasiliensis*. Journal of Phytopathology **117**: 373-376

Gilchrist D (1998) Programmed cell death in plant disease: the purpose and promise of cellular suicide. Annual Review of Phytopathology **36**: 393-414

Gille G and Sigler K (1995) Oxidative stress and living cells. Folia Microbiologica **40**: 131-152

Glazener J, Orlandi E and Baker C (1996) The active oxygen response of cell suspensions to incompatible bacteria is not sufficient to cause hypersensitive cell death. Plant Physiology **110**: 759-763

Gloria L and Uritani I (1984) Changes in ß-carotene content of golden yellow cassava in relation to physiological deterioration. *In* Uritani I and Reyes (eds.) Tropical Root Crops: Postharvest Physiology and Processing JSSP. Tokyo pp 163-168.

Gómez-Vásquez R, Day RC, Beeching JR and Cooper RM (1998) Biochemical components of disease resistance in cassava. IVth Scientific Meeting of Cassava Biotechnology Network, Salvador, Brazil, Nov, 5-13.

Gonzalez A, Schopke C, Taylor N, Beachy R and Fauquet C (1998) Regeneration of transgenic cassava plants (*Manihot esculenta* Crantz) through *Agrobacterium*-mediated transformation of embryogenic suspension cultures. Plant Cell Reports **17**: 827-831

Goodman R, Kiraly Z and Wood K (1986) The biochemistry and physiology of plant disease. University of Missouri Press, Columbia, USA

Greenberg J (1996) Programmed cell death: a way of life for plants. Proceedings of the National Academy of Sciences of the USA **93**: 12094-12097

Groom Q, Torres M, Fordham-Skelton A, Hammond-Kosack K, Robinson N and Jones J (1996) rbohA, a rice homologue of the mammalian gp91phox respiratory burst oxidase. Plant Journal **10**: 515-522

Guan L, Polidoros A and Scandalios J (1996) Isolation, characterization and expression of the maize *Cat2* catalase gene. Plant Molecular Biology **30**: 913-924

Guan L and Scandalios J (1993) Characterization of the catalase antioxidant defense gene *Cat1* of maize, and its developmentally regulated expression in transgenic tobacco. Plant Journal **3**: 527-536

Guan L and Scandalios J (1995) Developmentally related responses of maize catalase genes to salicylic acid. Proceedings of the National Academy of Sciences USA **92**: 5930-5934

Guan L, Scandalios J (1996) Molecular evolution of maize catalases and their relationship to other eukaryotic and prokaryotic catalases. Journal of Molecular Evolution **42**: 570-579

Hall J, Flowers T, Roberts R (1992) Plant cell structure and metabolism. Second edition. Longman, London, UK

Han Y (2000) Molecular analysis of post-harvest physiological deterioration. PhD thesis. University of Bath.

Han Y, Gomez-Vasquez R, Reilly K, Li H, Tohme J, Cooper R and Beeching J (2000) Hydroxyproline-rich glycxoproteins expressed during stress responses in cassava. Euphytica. *In press*

Hayashi M, Aoki M, Kondo M, Nishimura M. (1997) Changes in targeting efficiencies of proteins to plant microbodies by amino acid substitutions in the carboxy terminal tripeptide. Plant Cell Physiology **38**: 759-768

Heimgartner U, Pietrzaket M, Geertsen R, Brodelius P, da Silva Figueredo A, and Paiss M (1990) Purification and partial characterization of milk clotting proteases from flowers of *Cynara cardunculus*. Phytochemistry **29**: 1405-1410

Higo K and Higo H (1996) Cloning and characterisation of the rice *CatA* catalase gene, a homologue of the maize *Cat3* gene. Plant Molecular Biology **30**: 505-521

Hirose S, Data E and Quevedo M (1984a) Changes in respiration and ethylene production in cassava roots in relation to postharvest deterioration. *In* Uritani I and Reyes ED (eds.) Tropical Root Crops: Postharvest Physiology and Processing, Japan Scientific Societies Press, Tokyo. pp 83-98

Hirose S, Data E and Maturan E (1984b) Relation of respiration and ethylene production to postharvest deterioration in cassava roots from pruned and unpruned plants. *In* Uritani, I and Reyes, E (eds.) Tropical Root Crops: Postharvest Physiology and Processing Japan Scientific Societies Press: Tokyo

Hirose S and Data E (1984) Physiologoy of postharvest deterioration of cassava roots. *In* Uritani, I and Reyes, E (eds.) Tropical Root Crops: Postharvest Physiology and Processing Japan Scientific Societies Press: Tokyo. pp 37-51

Hirose S (1986) Physiological studies on postharvest deterioration of cassava plants. Japan Agricultural Research Quarterly **19**: 241-252

Hondt KD, Stack S, Gutteridge S, Vandekerckove J, Krebbers E and Gal S (1997) Aspartic protease genes in the Brassicaceae *Arabidopsis thaliana* and *Brassica napus*. Plant Molecular Biology **33**: 187-192

Huang J, Bachem C,Vermeesch A, Suurs L, Jacobson E and Visser R (2000) Analysis of post harvest deterioration in cassava. *In* Carvalho L, Thro A, and Vilarinhos A (eds.) Cassava Biotechnology. Proceedings of the 4th International Scientific Meeting, Salvador, Brazil 1998, EMBRAPA, Brasilia pp 537-550

Hughes J and Hughes MA (1994) Multiple secondary plant product UDP-glucose glucosyltransferase genes expressed in cassava (*Manihot-esculenta* Crantz) cotyledons. DNA Sequence **5**: 41-49.

Hughes J, Keresztessy Z, Brown K, Suhandono S and Hughes MA (1998) Genomic organization and structure of alpha-hydroxynitrile lyase in cassava (*Manihot esculenta* Crantz). Archives of Biochemistry and Biophysics **356**: 107-116.

Hunt L, Whorley D and Cock J (1977) Growth physiology of cassava. Field Crop Abstracts **30**: 77-91

Hussain A, Bushuk W, Ramirez H and Roca W (1987) Identification of cassava (*Manihot esculenta* Crantz) cultivars by electrophoretic patterns of esterase isozymes. Seed Science Technology **15**: 19-22

Ibrahim R, Benesi M, Issa J and Mkumbira J (1998) Opportunities and success of biotechnology methods in Malawi. Revista Brasileira de Mandioca **17**: 49

Iglesias C, Hershey C, Calle F and Bolanos A (1994) Propagating cassava (*Manihot esculenta*) by sexual seed. Experimental Agriculture **30**: 283-290

Iglesias C, Mayer JE, Chaves AL and Calle F (1995) Exploring the genetic potential and stability of β-carotene content in cassava roots. Biotechnology Research Unit Annual Report 1995, CIAT, Cali pp33-38

Iglesias C, Mayer J, Chavez L and Calle F (1997) Genetic potential and stability of carotene content in cassava roots. Euphytica **94**: 367-373

Iglesias C, Bedoya J, Morante N and Calle F (1996) Genetic diversity for physiological deterioration in cassava roots. *In* Kurup G, Palaniswami M, Potty V, Padmaja G,

Kabeerathumma S and Pillai S (eds.) Tropical Tuber Crops: Problems, Prospects and Future Strategies. Oxford & IBH Publishing Company, New Delhi pp 73-81

Ishige F, Mori H, Yamazaki K and Imaseki H (1993) Identification of a basic glycoprotein induced by ethylene in primary leaves of azuki bean as a cationic peroxidase. Plant Physiology **101**: 193-199

Iwamoto M, Maekawa M, Saito A, Higo H, and Higo K (1998) Evolutionary relationship of plant catalase genes inferred from exon-intron structures: isozyme divergence after the separation of monocots and dicots. Theoretical and Applied Genetics **97**: 9-19

Jabs T, Tschope M, Colling C, Hahlbrook K and Scheel D (1997) Elicitor stimulated ion fluxes and reactive oxygen species from the oxidative burst- signal defence gene activation and phytoalexin synthesis in parsley. Proceedings of the National Academy of Sciences of the USA **94**: 4800-4805

Jennings D (1976) Cassava *Manihot esculenta* (Euphorbiaciae) *In* Evolution of crop plants, Simmonds N (ed) pp 81-84

John I, Hackett R, Cooper W, Drake R, Farrell A, and Grierson D (1997) Cloning and characterisation of tomato leaf senescence related cDNAs. Plant Molecular Biology **33**: 641-651

Jones J and Mullet J (1995) A salt and dehydration inducible pea gene, *Cyp*15a, encodes a cell wall protein with sequence similarity to cysteine proteases. Plant Molecular Biology **28**: 1055-1065

Jones M, Larson P and Woodson W (1995) Ethylene regulated expression of a carnation cysteine protease during flower petal senescence. Plant Molecular Biology **28**: 505-512

Joshi B, Sainani M, Bastawade K, Gupta V and Ranjekar P (1998) Cysteine protease inhibitor from pearl millet: a new class of antifungal protein. Biochemical and Biophysical Research Communications **246**: 382-387

Joshi C, Zhou H, Huang X, and Chiang V (1997) Context sequences of translation initiation codon in plants. Plant Molecular Biology **35**: 993-1001

Kahn V (1977) Latency properties in avocado cultivars differing in their rate of browning. Journal of Science Food and Agriculture **28**: 233-239

Kang Hand Hershey J (1994) Effect of initiation factor eIF-5A depletion on protein synthesis and proliferstion of *Saccharomyces cerevisiae*. Journal of Biological Chemistry **269**: 3934-3940

Kardialsky I and Brewin N (1996) Expression of cysteine protease genes in pea nodule development and senescence. Molecular Plant Microbe Interactions **9**: 689-695

Kato M, Carvalho V and Correa H (1991) Efeitos da poda na deterioracao fisioloica, atividade enzimatica e nos teores de compostos fenolicos em raizes de mandioca. Pesquisa Agropecuaria Brasileira **26**: 237-245

Kissil J and Kimchi A (1998) Death associated proteins: from gene identification to the analysis of their apoptotic and tumour suppressive functions. Molecular Medicine Today **4**: 268-274

Kervinen J, Kontturi M and Mikola J (1990) Changes in the proteinase composition of barley leaves during senescence in field conditions. Cereal Research Communications **18**: 191-197

Kervinen J, Tobin G, Costa J, Waugh D, Wlodawer A and Zdanov A (1999) Crystal structure of plant aspartic protease phytepsIn inactivation and vacuolar targeting. EMBO Journal **18**: 3947-3955

Kliebenstein D, Monde R and Last R (1998) Superoxide dismutase in *Arabidopsis*: An eclectic enzyme family with disparate regulation and protein localization. Plant Physiology **118**: 837-650

Klotz M, Klassen G and Loewen P (1997) Phylogenetic relationships among prokaryotic and eukaryotic catalases. Molecular Biology and Evolution **14**: 951-958

Koleske A and Young R (1995) The RNA polymerase II holoenzyme and its implications for gene regulation. Trends in Biochemical Sciences **20**: 113-116

Kouzuma Y, Kawano K, Kimura M, Yamasaki N, Kadowaki T and Yamamoto K (1996) Purification, characterisation and sequencing of two cysteine proteinase inhibitors, Sca and Scb, from sunflower (*Helianthus annuus*) seeds. Journal of Biochemistry **119**: 1106-1113

Kozak M (1986) Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44**: 283-292

Kpemoua K, Boher B, Nicole M, Calatayud P and Geiger JP (1996) Cytochemistry of defense responses in cassava infected by *Xanthomonas campestris* pv. *manihotis*. Canadian Journal Of Microbiology **42**: 1131-1143

Lacan D, Baccou JC (1998) High levels of antioxidant enzymes correlate with delayed senescence in nonnetted muskmelon fruits. Planta **204**: 377-382

Lagrimini L, Gingas V, Finger F, Rothstein S and Liu T. (1997) Characterization of antisense transformed plants deficient in the tobacco anionic peroxidase. Plant Physiology **114**: 1187-1196

Lalaguna F and Agudo M (1989) Relationship between changes in lipid with ageing of cassava roots and senescence parameters. Phytochemistry **28**: 2059-2062

Lancaster PA and Brooks JE (1983) Cassava leaves as human food. Economic Botany **37**: 331-348.

Larson R (1988) The antioxidants of higher plants. Phytochemistry **27**: 969-978

Larson R (1995) Plant defenses against oxidative stress. Archives of Insect Biochemistry and Physiology **29**: 175-186

Laukkanen H, Haggman H, Kontunen-Soppela S and Hohtola A (1999) Tissue browning of in vitro cultures of Scots pine: role of peroxidase and polyphenol oxidase. Physiologia Plantarum **106**: 337-343

Lea P and Leagood R (1999) Plant Biochemistry and Molecular Biology, Second edition, Wiley and Sons, UK

Ledias F, Rangel P and Hansberg W (1998) Oxidation of catalase by singlet oxygen. Journal of Biological Chemistry **273**: 10630-10637

Lee H, Kim K, You S, Kwon S and Kwak S (1999) Molecular characterisation and expression of a cDNA encoding copper/zinc superoxide dismutase from cultured cells of cassava. Molecular and General Genetics **262:** 807-814

Lefevre F and Charrier A (1993) Heredity of seventeen isozyme loci in cassava (*Manihot esculenta* Crantz) Euphytica **66**: 171-178

Lefèvre F and Charrier A (1993) Isozyme diversity with African *Manihot* germplasm. Euphytica **66**: 73-80

Legendre L, Reuter S, Heinstein P and Low P (1993) Characterisation of the oligogalacturonide induced oxidative burst in cultured soybean cells. Plant Physiology **102**: 233-240

Leon J, Lawton M and Raskin I (1995) Hydrogen peroxide stimulates salicylic acid biosynthesis in tobacco. Plant Physiology **108**: 1673-1678

Levine A, Pennell R, Alvarez M, Palmer R and Lamb C (1996) Calcium mediated apoptosis in a plant hypersensitive disease resistance response. Current Biology **6**: 427-437

Lewin B (1997) Genes VI. Oxford University Press.

Li X, Sohly H, Nimrod A and Clark A (1999) Antifungal activity of epigallocatechin gallate from *Coccoloba dugandiana.* Planta Medica **65**: 780

Li H, Han Y and Beeching JR (2000) Isolation and characterisation of an ACC oxidase gene from cassava. *In* Carvalho L, Thro A and Vilarinhos A (eds.), Cassava Biotechnology. Proceedings of the 4th International Scientific Meeting, Salvador, Brazil 1998. Brasilia pp 582-589

Lidgett A, Moran M, Wong K, Furze J, Rhodes M and Hamill J (1995) Isolation and expression pattern of a cDNA encoding a cathepsin B like protease from *Nicotiana rustica.* Plant Molecular Biology **29**: 379-384

Lindholm P, Kuittinen T, Sorri O, Guo D, Merits A, Tormakangas K and Roonberg-Roos P (2000) Glycosylation of phytepsin and expression of dad1, dad2 and ost1 during onset of cell death in germinating barley scutella. Mechanisms of Development **93**: 169-173

Linthorst M, van der Does C, Brederode F and Bol JF (1993) Circadian expression and induction by wounding of tobacco genes for cysteine proteinase. Plant Molecular Biology **21**: 685-694

Liu Z and Ger M (1997) Changes of enzyme activity during pollen germination of maize and possible evidence of lignin synthesis. Australian Journal of Plant Physiology **24**: 329-335

Lohman KN, Gan S, Manorama J and Amasino R (1994) Molecular analysis of natural leaf senescence in *Arabidopsis thaliana.* Physiologia Plantarum **92**: 322-328

Low P and Merida J (1996) The oxidative burst in plant defence: function and signal transduction. Physiologia Plantarum **96**: 533-542

Mader M, Ungemach J and Schlos P (1980) The role of peroxidase isoenzyme groups of *Nicotiana tabacum* in hydrogen peroxide formation. Planta **147**: 467-470

Mader M and Amberg-Fisher V (1982) Role of peroxidase in lignification of tobacco cells: oxidation of nicotinamide adenine dinucleotide and formation of hydrogen peroxide by cell wall peroxidases. Plant Physiology **70**: 1128-1131

Mader M (1992) Compartmentation of plant peroxidase isoenzymes in plant cells. *In* Penel C, Gaspar T and Greppin H (eds.) Plant Peroxidases 1980-1990, Topics and Detailed Literature on Molecular, Biochemical and Physiological Aspects, University of Geneva

Malone M (1996) Rapid, long-distance signal transmission in higher plants. Advances in Botanical Research **22**: 163-228.

Manchenko G (1994) Handbook of Detection of Enzymes on Electrophoretic Gels, CRC Press, Boca Raton

Marriott J, Been B and Perkins C. (1978) The aetiology of vascular discoloration in cassava roots after harvesting: association with water loss from wounds. Physiologia Plantarum **44**: 38-42

Marriott J, Plumbley R and Rickard J (1980) Physiological aspects of the storage of cassava and other tropical root crops. *In* Hurd R, Biscoe P and Dennis C (eds.) Opportunities for Increasing Crop Yields. Pitman, London pp 363-375

Martilla S, Jones B and Mikkonen A (1995) Differential localisation of two acid proteinases in germinating barleyseed.. Physiologia Plantarum **68**: 282-286

Mas P and Pallas V (1995) Non isotopic tissue print hybridization, a new technique to study long distance plant virus movement. Journal of Virological Methods **52**: 317-326

May M, Hammond-Kosack K and Jones J (1996) Involvement of reactive oxygen species, glutathione metabolism, and lipid peroxidation in the Cf gene dependant defence response of cotton cotyledons induced by race specific elicitors of *Cladopsporium fulvum.* Plant Physiology **110**: 1367-1379

McCarthy C (1997) Chromas – user manual and latest version freeware. http://trishul.sci.gu.edu.au/~conor/chromas.html

McClung C (1997) Regulation of catalases in *Arabidopsis*. Free Radical Biology and Medicine **23**: 489-496

McDowell J and Dangl J (2000) Signal transduction in the plant immune response. Trends in Biological Science **25**: 79-82

McKune K, Moore P, Hull M and Woychik N (1995) Six human RNA polymerase subunits functionally substitute for their yeast counterparts. Molecular and Cellular Biology **15**: 6895-6900

Mehdy M, Sharma Y, Sathasivan K and Bays N (1996) The role of activated oxygen species in plant disease resistance. Physiologia Plantarum **98**: 365-374

Miao Z and Gaynor J (1993) Molecular cloning characterisation and expression on Mn superoxide dismutases from rubber tree. Plant Molecular Biology **23**: 267-277

Michaud D, Nguyen-Quoc B and Yelle S (1993) Selective inhibition of Colorado potato beetle cathepsin H by oryzacystatins I and II. FEBS Letters **331**: 173-176

Milosovic N and Slusarenko A (1996) Active oxygen metabolism and lignification in the hypersensitive response in bean. Physiological and Molecular Plant Pathology **49**: 143-158

Mithofer A, Daxberger A, Fromhold-Treu D and Ebel J (1997) Involvement of an NADPH oxidase in the elicitor inducible oxidative burst of soybean. Phytochemistry **45**: 1101-1107

Miura Y, Yoshioka H and Doke N (1995) An autoradiographic determination of the active oxygen generation in potato tuber disks during hypersensitive response to fungal infection or elicitor. Plant Science **105**: 45-52

Montaldo A (1973) Vascular streaking of cassava root tubers. Tropical Science **1**: 39-46

Mullen R, Lee M, Trelease R (1997) Identification of the peroxisomal targeting signal for cottonseed catalase. Plant Journal **12**: 313-322

Murphy A, Chivasa S, Singh D and Carr J (1999) Salicylic acid-induced resistance to viruses and other pathogens: a parting of the ways? Trends in Plant Science **4**: 155-160

Mutlu A and Gal S (1999) Plant aspartic proteinases: enzymes on the way to function. Physiologia Plantarum **105**: 569-576

Nakai K (2000) PSORT users manual. http://psort.nibb.ac.jp/helpwww.html

Nair S, Jos J and Lakshmi K (1996) Carotene enhancement in cassava through gene pool development. *In*: Kurup G, Palaniswami M, Potty V, Padmaja G, Kabeerathumma S and Pillai S (eds.) Tropical Tuber Crops: Problems, Prospects and Future Strategies,. Oxford & IBH Publishing Company, New Delhi pp 82-87

Nicholas K and Nicholas H (1997) GeneDoc: a tool for editing and annotating multiple sequence alignments. Distributed by the author. www.cris.com/~ketchup/genedoc.shtml

Nielsen H, Engelbrecht J, Brunak S and von Heijne G (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Engineering **10**: 1-6

Noon R, and Booth R (1977) Nature of post-harvest deterioration of cassava roots. Transactions of the British Mycological Society **69**: 287-290

Ollinger K (2000) Inhibition of Cathepsin D prevents free radical induced apoptosis in rat cardiomyocytes. Archives of Biochemistry and Biophysics **373**: 346-351

Olsen L (1998) The surprising complexity of peroxisome biogenesis. Plant Molecular Biology **38**: 163-189

Olson P and Varner J (1993) Hydrogen peroxide and lignification. Plant Journal **4**: 887-892

Olson K and Schaal B (1999) Evidence on the origin of cassava: Phylogeography of *Manihot esculenta*. Proceedings of the National Academy of Science **96**: 5586-5591

Onwueme I (1978) The Tropical Tuber Crops. John Wiley, Chichester.

Orozco-Cardenas M and Ryan C (1999) Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. Proceedings of the National Academy of Sciences USA **96**: 6553-6557

Ossowoski I, Hausner G and Loewen P (1993) Molecular evolutionary analysis based on the amino acid sequence of catalase. Journal of Molecular Evolution **37**: 71-76

Ostergaard L; Pedersen A, Jespersen H, Brunak S and Welinder K (1998) Computational analyses and annotations of the *Arabidopsis* peroxidase gene family. FEBS Letters **433**: 98-102

Ota Y, Ario T, Hayashi K, Nakagawa T, Hattori T, Maeshima M and Asahi T (1992) Tissue specific isoforms of catalase subunits in castor bean seedlings. Plant Cell Physiology **33**: 225-232

Padmaja G and Balagopal C (1985) Cellular and extracellular enzymes associated with the post harvest deterioration of cassava tubers. Journal of Food Science and Technology **22**: 82-87

Panavas T and Rubenstein B (1998) Oxidative events during programmed cell death of Daylily petals. Plant Science **133**: 125-138

Panavas T, Pikula A, Reid P, Rubinstein B and Walker E (1999) Identification of senescence-associated genes from daylily petals. Plant Molecular Biology **40**: 237-248

Passan HC and Noon RA (1977) Deterioration of yams and cassava during storage. Annals of Applied Biology **85**: 436-440

Pay A, Heberle-Bors E and Hirt H (1991) Isolation and sequence determination of the plant homologue of the eukaryotic initiation factor 4D cDNA from alfafa, *Medicago sativa*. Plant Molecular Biology **17**: 927-929

Pernas M, Sanchez-Monge R, Gomez L and Salcedo G (1998) A chestnut seed cystatin differentially effective against cysteine proteinases from closely related pests. Plant Molecular Biology **38**: 1235-1242

Petit-Paly G, Franck T, Brisson L, Kevers C, Chenieux J and Rideau M (1999) Cytokinin modulates catalase activity and coumarin accumulation in *in vitro* cultures of tobacco. Journal of Plant Physiology **155**: 9-15

Peyrado G, Forchetti S, Tigier H and Taleisnik E (1996) Tissue printing for peroxidases associated with lignification. Biotechnic and Histochemistry **71**: 258-262

Pita J, Fondong V, Sangar A, Otim-Nape G, Ogwall S and Fauquet C (2000) Biodiversity of cassava mosaic disease in east and west Africa. *In* Carvalho L, Thro A and Vilarinhos A (eds.), Cassava Biotechnology. Proceedings of the 4th International Scientific Meeting, Salvador, Brazil 1998. Brasilia 2000 pp 299-307

Pitcher L and Zilinskas B (1996) Overexpression of copper/zinc superoxide dismutase in the cytosol of transgenic tobacco confers partial resistance to ozone induced foliar necrosis. Plant Physiology **110**: 583-588

Piperno D and Holst I (1998) The presence of starch grains on prehistoric stone tools from the humid neotropics: Indications of early tuber use and agriculture in Panama. Journal of Archaeological Science **25**: 765-776.

Plumbley R, Hughes P, and Marriott J. (1981) Studies on peroxidases and vascular deterioration in cassava root tissue. Journal of Science in Food and Agriculture **32**: 723-731

Plumbley R and Hughes P (1982) DEAE-cellulose separation of peroxidases from cassava (*Manihot esculenta* Crantz) root-tissue. Journal of Food Biochemistry **6**: 197-206

Plumbley R and Rickard J (1991) Post-harvest deterioration of cassava. Tropical Science **31**: 295-303

Pontier D, Gan S, Amasino R, Roby D and Lam E (1999) Markers for hypersensitive response and senescence show distinct patterns of expression. Plant Molecular Biology **39**: 1243-1255

Puonti-Kaerlas J (1998) Cassava biotechnology. Biotechnology and Genetic Engineering Reviews **15**: 329-364

Quiroga M, Guerrero C, Botella M, Barcelo A, Amaya I, Medina M, Alonso F, Forchetti S, Tigier H and Valpuesta V (2000) A tomato peroxidase involved in the synthesis of lignin and suberin. Plant Physiology **122**: 1119-1127

Ramalho-Santos M, Verissimo P, Cortes L, Samyn B, Van Beeumen J, Pires E, Faro C (1998) Identification and proteolytic processing of procardosin A. European Journal of Biochemistry **225**: 133-138

Rau M, Paliyath G, Ormrod D, Murr D and Watkins C (1997) Influence of salicylic acid on H_2O_2 production, oxidative stress and H_2O_2 metabolising enzymes. Plant Physiology **115**: 137-149

Raught B and Gingras A (1999) eIF4E activity is regulated at multiple levels. Jorunal of Biochemistry and Cell Biology **31**: 43-57

Ravi V, Aked J and Balagopalan C (1996) Review on tropical root and tuber crops .1. Storage methods and quality changes. Critical Reviews in Food Science and Nutrition **36**: 661-709

Rawlings N and Barret A (1995) Families of aspartic peptidases, and those of unknown catalytic mechanism. Methods in Enzymology **248**: 105-120

Reid T, Murthy M, Sicignano A, Tanaka N, Musick W and Rossmann M. (1981) Structure and heme environment of beef liver catalase at 2.5A resolution. Proceedings of the National Academy of Sciences of the USA **78**: 4767-4771

Reilly K, Han Y, Tohme J, Beeching JR (2000) Oxidative stress related genes and cassava post-harvest physiological deterioration. *In* Carvalho L, Thro A and Vilarinhos

A (eds.), Cassava Biotechnology. Proceedings of the 4th International Scientific Meeting, Salvador, Brazil 1998. Brasilia pp 560-571

Rice-Evans C (1995) Plant polyphenols - free-radical scavengers or chain-breaking antioxidants. Biochemical Society Symposium **61**: 103-116

Rickard J (1982) Investigations into post-harvest behavior of cassava roots and their responses to wounding. PhD thesis. University of London

Rickard J, Marriott J and Gahan P (1979) Occlusions in cassava xylem vessels associated with vascular discoloration. Annals of Botany **43**: 523-526

Rickard J (1985) Physiological deterioration of cassava roots. Journal of Food and Agriculture **36**: 167-176

Rickard J and Gahan P (1983) The development of occlusions in cassava root xylem vessels. Annals of Botany **52**: 811-821

Roberts E, Kutchen T and Kolattukudy P (1988) Cloning and sequencing of cDNA for a highly anionic peroxidase from potato and the induction of its mRNA in suberizing potato tubers and tomato fruit. Plant Molecular Biology **11**: 15-26

Rodriguez M, Buschmann H, Tohme J and Beeching J (2000) Production of anti-microbial compounds in cassava (*Manihot esculenta* Crantz) root during post-harvest physiological deterioration. *In* Carvalho L, Thro A and Vilarinhos A (eds.) Cassava Biotechnology. Proceedings of the 4th International Scientific Meeting, Salvador, Brazil 1998. Brasilia 2000 pp 320-328

Roeder R and Rutter W (1969) Multiple forms of DNA-dependant RNA polymerase in eukaryotic organisms. Nature **224**: 234-237

Rogers D and Appan S (1973) Flora Neotropica Monograph No 13: Manihot Manhotoides (Euphorbiaceae), New York, Hafner Press

Roonberg Roos P and Saarma M (1998) Phytepsin, a barley vacuolar aspartic proteinase, is highly expressed during autolysis of developing tracheary elements and sieve cells. Plant Journal **15**: 139-145

Ros Barcelo A. (1998) The generation of H_2O_2 in the xylem of *Zinnia elegans* is mediated by an NADPH-oxidase like enzyme. Planta **207**: 207-216

Rosling H (1998) Cassava cyanogenesis Plenary session 2 Fourth International Meeting Cassava Biotechnology Network, 3-7 November 1998, Salvador, Bahia, Brazil

Ross A, Manners J and Birch R (1995) Molecular cloning and characterization of peroxidases from buffel grass (*Cenchrus ciliaris* L.) Plant Science **110**: 95-103

Ruffer M, Steipe B, and Zenk M (1995) Evidence against specific binding of salicylic acid to plant catalase. FEBS Letters **377**: 175-180

Ryals J, Lawton K, Delany T, Freidrich L, Kessmann H, Neuenschwander U, Uknes S, Vernooij B, and Weymann K (1995) Signal transduction in systemic acquired resistance. Proceedings of the National Academy of Sciences of the USA **92**: 4202-4205

Ryan S, Laing W and McManus M (1998) A cysteine protease inhibitor purified from apple fruit. Phytochemistry **49**: 957-963

Sakai T, Nakagawa Y, Uritani I and Data ES (1986) Occurrence of various kinds of metabolites in physiologically and microbially damaged cassava (*Manihot esculenta* Crantz) roots. Agric. Biol. Chem. **50**: 2905-2907.

Sakajo S, Nakamura K, and Asahi T (1987) Increase in catalase mRNA in wounded sweet potato tuberous root tissue. Plant Cell Physiology **28**: 919-924

Sakurai H and Ishihama A (1997) Gene organization and protein sequence of the small subunits of *Shizosaccharomyces pombe* RNA polymerase II. Gene **196**: 165-174

Sambrook J, Frisch E, Maniatis T (1989) Molecular cloning: a laboratory manual. Second edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Sanchez G, Restrepo S, Duque MC, Fregene M, Bonierbale M and Verdier V (1999) AFLP assessment of genetic variability in cassava accessions (*Manihot esculenta*) resistant and susceptible to the cassava bacterial blight (CBB). Genome **42**: 163-172

Sanchez-Casas P and Klessig DF (1994) A salicylic acid-binding activity and a salicylic acid-inhibitable catalase activity are present in a variety of plant species. Plant Physiology **106**: 1675-1679

Scandalios J (1983) Molecular varieties of isozymes and their role in studies on gene regulation and expression during eukaryote development. Isozymes: Current Topics in Biological and Medical Research **9**: 1-31

Scandalios J (1990) Response of plant antioxidant defense genes to environmental stress. Advances in Genetics **28**: 1-41

Scandalios J, Guan L and Polidoros A (1997) Catalases in plants: gene structure, properties, regulation, and expression. *In* Scandalios J (ed), Oxidative Stress and the Molecular Biology of Antioxidant Defences, Cold Spring Harbor Laboratory Press, Cold Spring Harbor pp 343-406

Scandalios JG (1997) Molecular genetics of superoxide dismutase in plants. *In* Scandalios J (ed) Oxidative Stress and the Molecular Biology of Antioxidant Defenses, CSHL Press, Cold Spring Harbor pp 527-568

Schaller A and Ryan C (1996) Molecular cloning of a tomato leaf cDNA encoding an aspartic proteinase, a systemic wound response protein. Plant Molecular Biology **31**: 1076-1077

Scheel D (1998) Resistance response physiology and signal transduction. Current Opinion in Plant Biology **1**: 305-310

Schmid M, Simpson D and Gietl C (1999) Programmed cell death in castor bean endosperm is associated with the accumulation and release of a cysteine endopeptidase

from ricinosomes. Proceedings of the National Academy of Sciences USA **96**: 14159-14164

Schopfer P (1994) Histochemical-demonstration and localization of H_2 O_2 in organs of higher-plants by tissue printing on nitrocellulose paper. Plant Physiology **104**: 1269-1275

Schopke C, Masona M, Taylor N, Carcamo R, Ho T, Beachy R and Fauquet C (2000) Production and characterization of transgenic transgenic cassava plants expressing the coat protein gene of cassava common mosaic virus. *In* Carvalho L, Thro A and Vilarinhos A (eds.), Cassava Biotechnology. Proceedings of the 4th International Scientific Meeting, Salvador, Brazil 1998. Brasilia pp 236-243

Schopke C, Taylor N, Carcamo R, Konan N, Marmey P, Henshaw G, Beachy R and Fauquet C (1996) Regeneration of transgenic cassava plants (*Manihot-esculenta* Crantz) from microbombarded embryogenic suspension-cultures. Nature Biotechnology **14**: 731-735

Shi X, Yin K, Zimolo Z, Stern A and Waxman L (1996) The subcellular distribution of eukaryotic translation factor, eIF-5A, in culture cells. Experimental Cell Research **225**: 348-356

Schreck R, Rieber P and Baeuerle P (1991) Reactive oxygen intermediates as apparently widely used messengers in the activation of NFκβ transcription factor and HIV-1. EMBO Journal **10**: 2247-2258

Scott I, Dat J, Lopez-Delgado H and Foyer C (1999) Salicylic acid and hydrogen peroxide in abiotic stress signalling in plants. Phyton **39**: 13-17

Simon P, Capelli N, Flach J, Overney S, Tognolli M, Penel C and Greppin H (1996) The peroxidase gene family of *Arabidopsis thaliana*. *In* Obinger C, Burner U, Ebermann R, Penel C and Greppin H (eds.) Plant Peroxidases: Biochemistry and Physiology. University of Geneva pp 179-183

Skadsen R and Scandalios J (1983) Evidence for processing of maize catalase 3 and purification of its messenger RNA aided by translation of antibody bound polysomes. Biochemistry **25**: 2027-2032

Skadsen R, Schulze-Lefert P and Herbst J (1995) Molecular cloning characterisation and expression analysis of two catalase isozyme genes in barley. Plant Molecular Biology **29**: 1005-1014

Smart C, Hosken S, Thomas H, Greaves J, Blair B and Schuch W (1995) The timing of maize leaf senescence and characterisation of senescence related cDNAs. Physiologia Plantarum **93**: 673-682

Solomon M, Belenghi B, Delledonne M, Menachem E and Levine A (1999) The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. The Plant Cell **11**: 431-443

Song Z and Steller H (2000) Death by design: mechanism and control of apoptosis. Trends in Genetics **15**: 49-52

Stoscheck C (1990) Increased uniformity in the response of the Coomasie blue G assay to different proteins. Analytic Biochemistry **184**: 111-116

Strack P, Waxman L and Fagan J (1996) ATP-stimulated degradation of oxidatively modified superoxide dismutase by cathepsin D in cardiac tissue extracts. Biochemical and Biophysical Research Communications **219**: 348-353

Stroeher V, Maclagan J and Good A (1997) Molecular cloning of a *Brassica napus* cysteine protease gene inducible by drought and low temperature stress. Physiologia Plantarum **101**: 389-397

Suarez M, Bernal J, Gutierrez J, Tohme J and Fregene M (2000) Developing expressed sequence tags (ESTs) from polymorphic transcript derived fragments (TDFs) in cassava. Genome **43**: 62-67

Suzuki M, Ario T, Hattori T, Nakamura K, and Asahi T (1994) Isolation and characterization of two tightly linked catalase genes from castor bean that are differentially regulated. Plant Molecular Biology **25**: 507-516

Tajima F and Nei M (1984) Estimation of evolutionary distance between nucleotide sequences. Molecular Biology and Evolution **1**: 269-285

Takahashi H, Chen Z, Du H, Liu Y and Klessig D (1997) Development of necrosis and activation of disease resistance in transgenic plants with severely reduced catalase levels. Plant Journal **11**: 993-1005

Takao T, Kitatani F, Wanatabe N, Yagi A and Sakata K (1994) A simple screening method for antioxidants and isolation of several antioxidants produced by marine bacteria from fish and shellfish. Bioscience, Biotechnology and Biochemistry **58**: 1780-1783

Tanaka Y, Data E, Hirose S, Taniguchi T and Uritani I (1983) Biochemical changes in secondary metabolites in wounded and deteriorated cassava roots. Agricultural and Biological Chemistry **47**: 693-700

Tanaka Y, Data E, Lape V, Villegas C, Gorgonio M, Hirose S and Uritani I (1984) Effect of pruning treatment on physiological deterioration in cassava roots. Agricultural and Biological Chemistry **47**: 739-743

Tang J and Wong R (1987) Evolution of the structure and function of aspartic proteases. Journal of Cellular Biochemistry **33**: 53-63

Taniguchi T and Data E (1984) The role of microorganisms in post-harvest deterioration of cassava roots. *In* Tropical Root Crops: Postharvest Physiology and Processing. Urtani I and Reyes R (eds.). JSSP: Tokyo. pp 53-60

Tenhaken R, Levine A, Brisson L, Dixon R and Lamb C (1995) Function of the oxidative burst in hypersensitive disease resistance. Proceedings of the National Academy of Sciences USA **92**: 4158-4163

Tenhaken R and Rubel C (1997) Salicylic acid is needed in hypersensitive cell death in soybean but does not act as a catalase inhibitor. Plant Physiology **115**: 291-298

Thompson J, Higgins D, Gibson T (1994) ClustalW: improving the sensitivity of multiple sequence alignments through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Research **22**: 4673-4680

Thompson J, Legge R and Barber R (1987) The role of free radicals in senescence and wounding. New Phytology **105**: 317-344

Thordal-Christensen H, Shang Z, Wei Y and Collinge D (1997) Subcellular localisation of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-mildew interaction. Plant Journal **11**: 1187-1194

Tome M and Gerner E (1997) Cellular eukaryotic initiation factor 5A content as a mediator of polyamine effects on growth and apoptosis.Biological Signals **6**: 150-156

Trelease R, Xie W, Lee M and Mullen R (1996) Rat liver catalase is sorted to peroxisomes by its C-terminal tripeptide Ala-Asn-Leu not by the internal Ser-Lys-Leu motif. European Journal of Cell Biology **71**: 248-258

Thro A and Fregene M (1998) Network impact and scientific advances in cassava biotechnology. Tropical Agriculture **75**: 230-237

Thro A, Fregene M, Taylor N, Raemakers K, Puonti-Kaerlas J, Schopke C, Visser R, Potrykus I, Fauquet C, Roca W and Hershey C (1997) Genetic biotechnologies and cassava-based development. CIAT policy document.

Thro A (1998) Issues in farmer participation for CBN. Revista Brasileira de Mandioca **17**: 81

Turk V and Bode W (1991) The cystatins: protein inhibitors of cysteine proteinases. FEBS Letters **285**: 213-219

Uritani I, Data E, Villegas R, Flores P and Hirose S (1983) Relationship between secondary metabolism changes in cassava root tissue and physiological deterioration. Agricultural and Biological Chemistry **47**: 1591-1598

Uritani I, Data E and Tanaka Y (1984) Biochemistry of postharvest deterioration of cassava and sweet potato roots. *In* Uritani I and Reyes R (eds.) Tropical Root Crops: Postharvest Physiology and Processing. JSSP, Tokyo, pp 61-75

Uritani I, Takeuchi W, Kojima Y, Sasaki M, Naito S, Nagata K and Garcia V (1992) Some properties of proteins in taro corms and cassava roots. Nippon Shokuhin Koryo Gakkaishi **39**: 945-950.

Vallelian-Bindschedler L, Schweizer P, Mosinger E and Metraux J (1998) Heat induced resistance in barley to powdery mildew (*Blumeria graminis* f.sp. *hordei*) is associated with a burst of active oxygen species. Physiological and Molecular Plant Pathology **52**: 185-199

Valpuesta V, Lange N, Guerrero C and Reid M (1995) Up regulation of a cysteine protease accompanies the ethylene insensitive senescence of daylily (*Hemerocallis*) flowers. Plant Molecular Biology **28**: 575-582

Van Camp W, Bowler C, Villarroel R, Tsang E, Van Montagu M and Inze D (1990) Characterisation of iron superoxide-dismutase cDNAs from plants obtained by genetic complementation in *Escherichia-coli*. Proceedings of the National Academy of Sciences USA **87**: 9903-9907

Van Camp W, Inze D and Van Montagu M (1997) The regulation and function of tobacco superoxide dismutases. Free Radical Biology and Medicine **23**: 515-520

Van Camp W, Van Montagu M and Inze D (1998) H_2O_2 and NO: redox signals in disease resistance. Trends in Plant Science **3**: 330-334

Van de Peer Y, De Wachter R (1994) TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Computer Applications in Bioscience **10**: 569-570

Varner J (1992) Tissue printing demonstration. Tissue printing. Academic Press

Varner J and Ye Z (1994) Tissue printing. FASEB Journal **8**: 378-384

Villa P, Kaufmann S and Earnshaw W (1997) Caspases and caspase inhibitors. Trends in Biological Science **22**: 388-392

Wallace G and Fry S (1999) Action of diverse peroxidases and laccases on six cell wall-related phenolic compounds. Phytochemistry **52**: 769-773

Wanatabe T and Sakai S (1998) Effect of active oxygen species and methyl jasmonate on expression of the gene for a wound inducible 1-aminocyclopropane-carboxylate synthase in winter squash (*Cucurbita maxima*). Planta **206**: 570-576

Wang H, Li J, Bostock R and Gilchrist D (1996) Apoptosis: a functional paradigm for programmed plant cell death induced by a host selective phytotoxin and invoked during development. Plant Cell **8**: 375-391

Warm E and Laties G (1982) Quantification of hydrogen peroxide in plant extracts by the chemiluminescence reaction with luminol. Phytochemistry **21**: 827-831

Welinder K (1985) Plant peroxidases: their primary, secondary and tertiary structures, and relation to cytochrome c peroxidase. European Journal of Biochemistry **151**: 497-504

Welinder K (1992) Superfamily of plant, fungal and bacterial peroxidases. Current Biology **2**: 388-393

Welinder K (1992) Plant peroxidases: structure function relationships. *In* Penel C, Gaspar T and Greppin H (eds.) Plant peroxidases 1980-1990, Topics and Detailed Literature on Molecular, Biochemical and Physiological Aspects. University of Geneva pp1-24

Wenham JE (1995) Post-harvest Deterioration of Cassava. A Biotechnological Perspective. FAO, Rome

Wheatley C (1980) Studies related with the nature of post-harvest physiological deterioration in cassava roots. CIAT Seminarios Internos SE-16-80

Wheatley C (1982) Studies on cassava (*Manihot esculenta* Crantz) root post-harvest deterioration. PhD Thesis. University of London

Wheatley C and Schwabe W (1985) Scopoletin involvement in post-harvest physiological deterioration of cassava root (*Manihot esculenta* Crantz). Journal of Experimental Botany **36**: 783-791

Wheatley C Fernandez F and Medina L (1989) Conservation of cassava roots in polythene bags. CIAT pp 33

Wheatley C and Best R (1991) How can traditional forms of nutrition be maintained in urban centers: the case for cassava. Entwicklung und Laendlicher Raum **91**: 13-16.

Wickham L and Wilson L (1988) Quality changes during long term storage of cassava roots in moist media. Tropical Science **28**: 79-84

Willekins H, Villarroel R, van Montagu M, Inze D and van Camp W (1994) Molecular identification of catalases from *Nicotinia plumbaginifolia.* FEBS Letters **352**: 70-83

Williams J, Bulman M, Huttly A, Phillips A and Neill S (1994) Characterisation of a cDNA from *Arabidopsis thaliana* encoding a potential thiol protease whose expression is induced independently by wilting and abscissic acid. Plant Molecular Biology **25**: 259-270

Williamson J, and Scandalios J (1993) Response of the maize catalases and superoxide dismutases to cercosporin containing fungal extracts: the pattern of catalase response in scutella is stage specific. Physiologia Plantarum **88**: 159-166

Wojtaszek P (1997) Oxidative burst: an early plant response to pathogen infection. Biochemical Journal **322**: 681-692

Woodward B, Steyn A, Jerico C and Thompson G (2000) Cassava- a method for the elimination of viruses. *In* Carvalho L, Thro A and Vilarinhos A (eds.), Cassava Biotechnology. Proceedings of the 4th International Scientific Meeting, Salvador, Brazil 1998. Brasilia pp3 36-344

Woychik N, Liao S, Kolodziej and Young R (1990) Subunits shared by eukaryotic nuclear RNA polymerases. Genes and Development **4**: 313-323

Woyinka A, Abegunde V and Adewusi S (1995) Nutrient content of young cassava leaves and assessment of their acceptance as a green vegetable in Nigeria. Plant Foods for Human Nutrition **47**: 21-28.

Yahraus T, Chandra S, Legendre L and Low P (1995) Evidence for a mechanically induced oxidative burst. Plant Physiology **109**: 1259-1266

Yano A, Suzuki K and Shinshi H (1999) A signalling pathway independent of the oxidative burst that leads to hypersensitive cell death in cultured tobacco cells includes a serine protease. Plant Journal **18**: 105-109

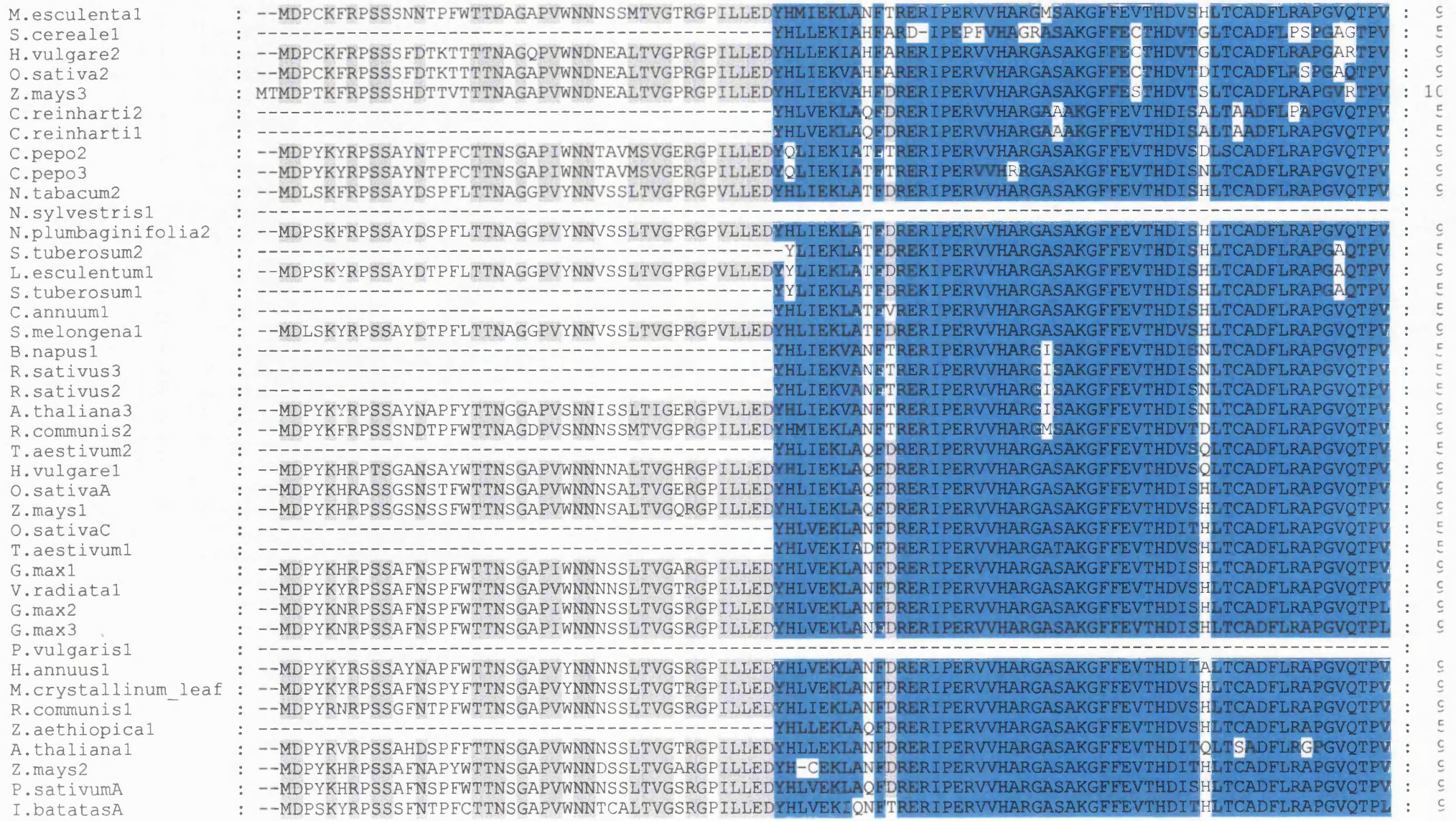
Ye Z and Varner J (1996) Induction of cysteine and serine proteases during xylogenesis in *Zinnia elegans*. Plant Molecular Biology **30**: 1233-1246

Yu D, Liu Y, Fan B, Klessig D and Chen Z (1997) Is the high basal level of salicylic acid important for disease resistance in potato? Plant Physiology **115**: 343-349

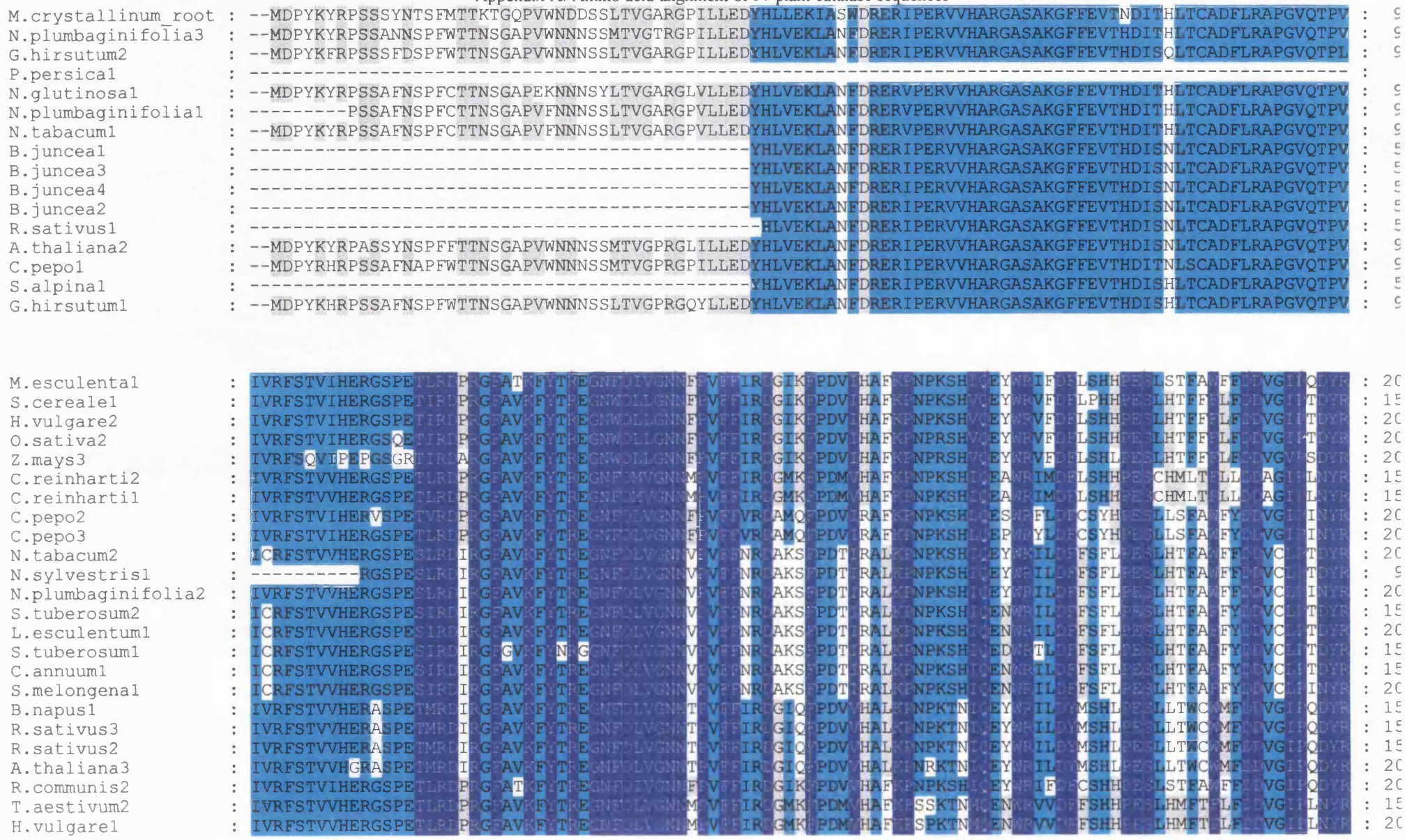
Zamocky M and Koller F (1999) Understanding the structure and function of catalases: clues from molecular evolution and in vitro mutagenesis. Progress in Biophysics and Molecular Biology **72**: 19-66

Zhu D and Scandalios J (1993) Maize mitochondrial superoxide dismutases are encoded by a differentially expressed multigene family. Proceedings of the National Academy of Sciences USA **90**: 9310-9314

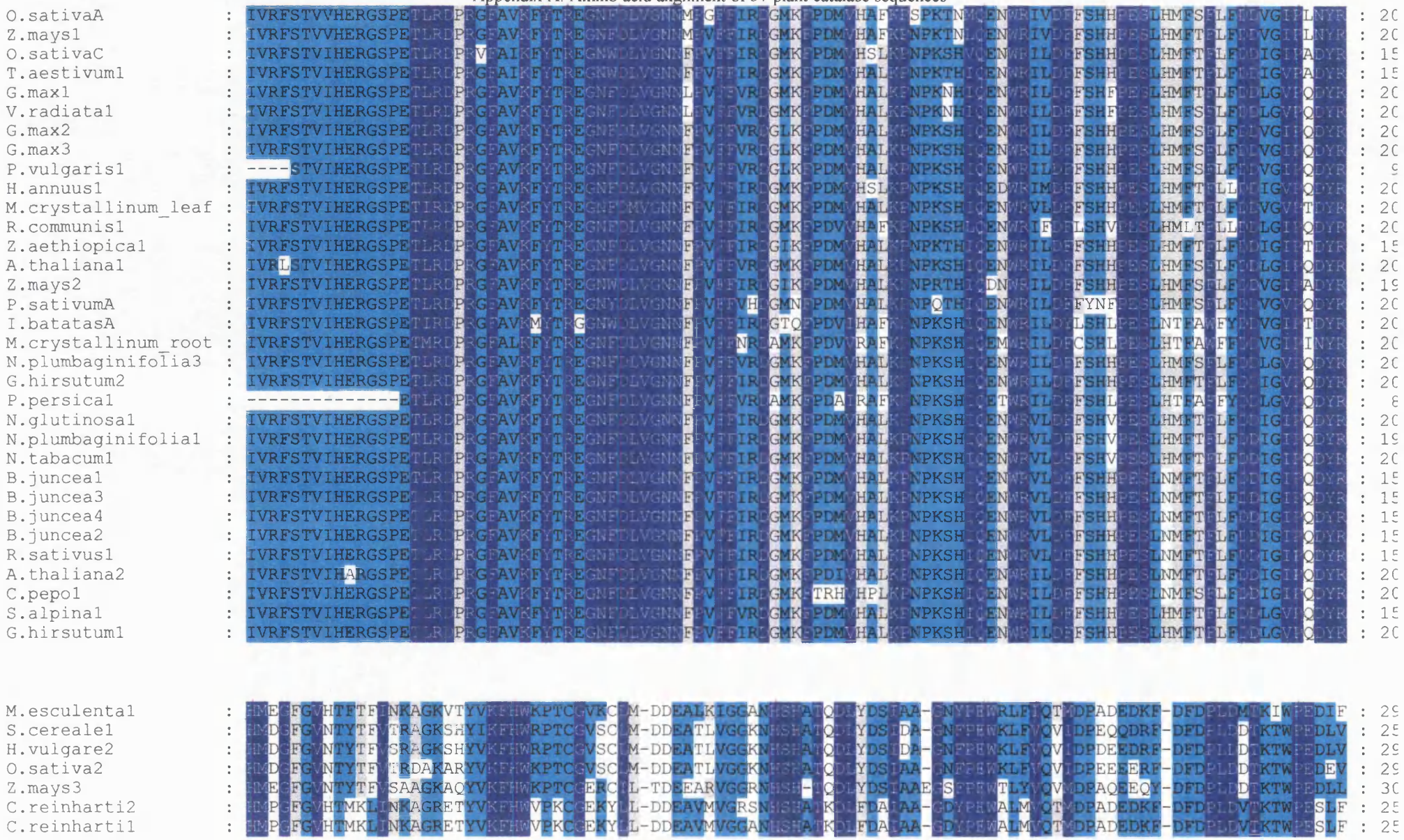
Appendix A. Amino acid alignment of 57 plant catalase sequences



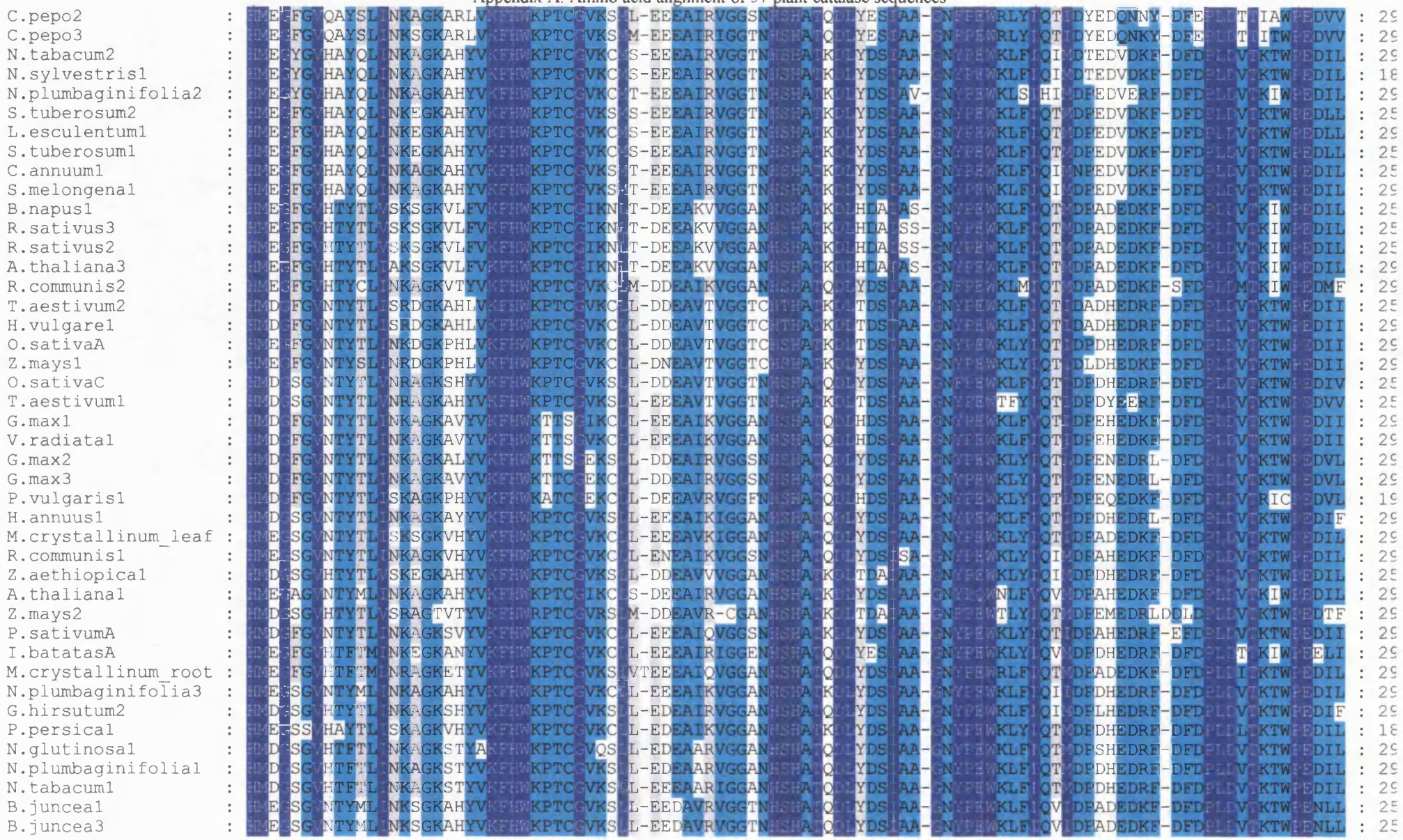
Appendix A. Amino acid alignment of 57 plant catalase sequences



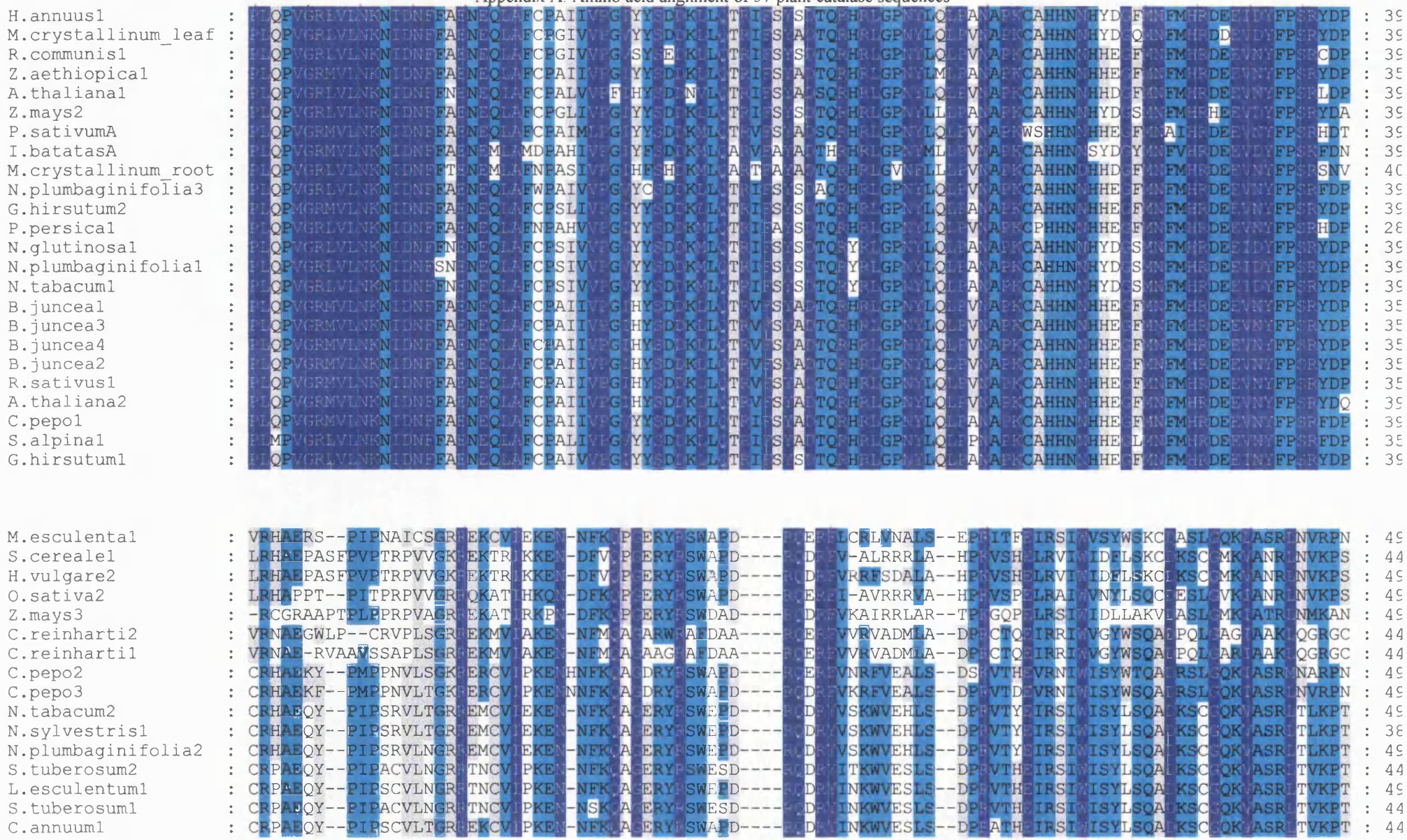
Appendix A. Amino acid alignment of 57 plant catalase sequences



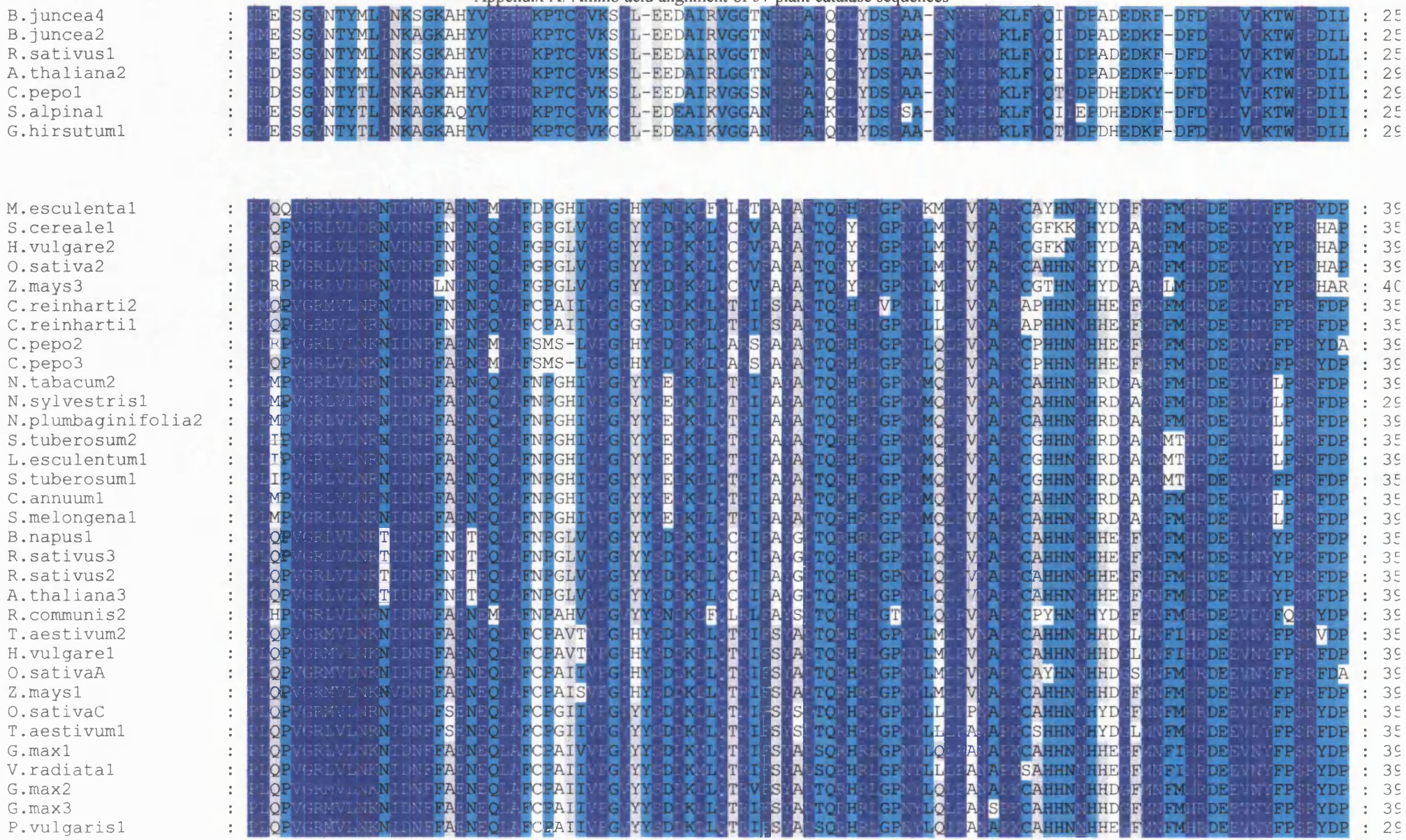
Appendix A. Amino acid alignment of 57 plant catalase sequences



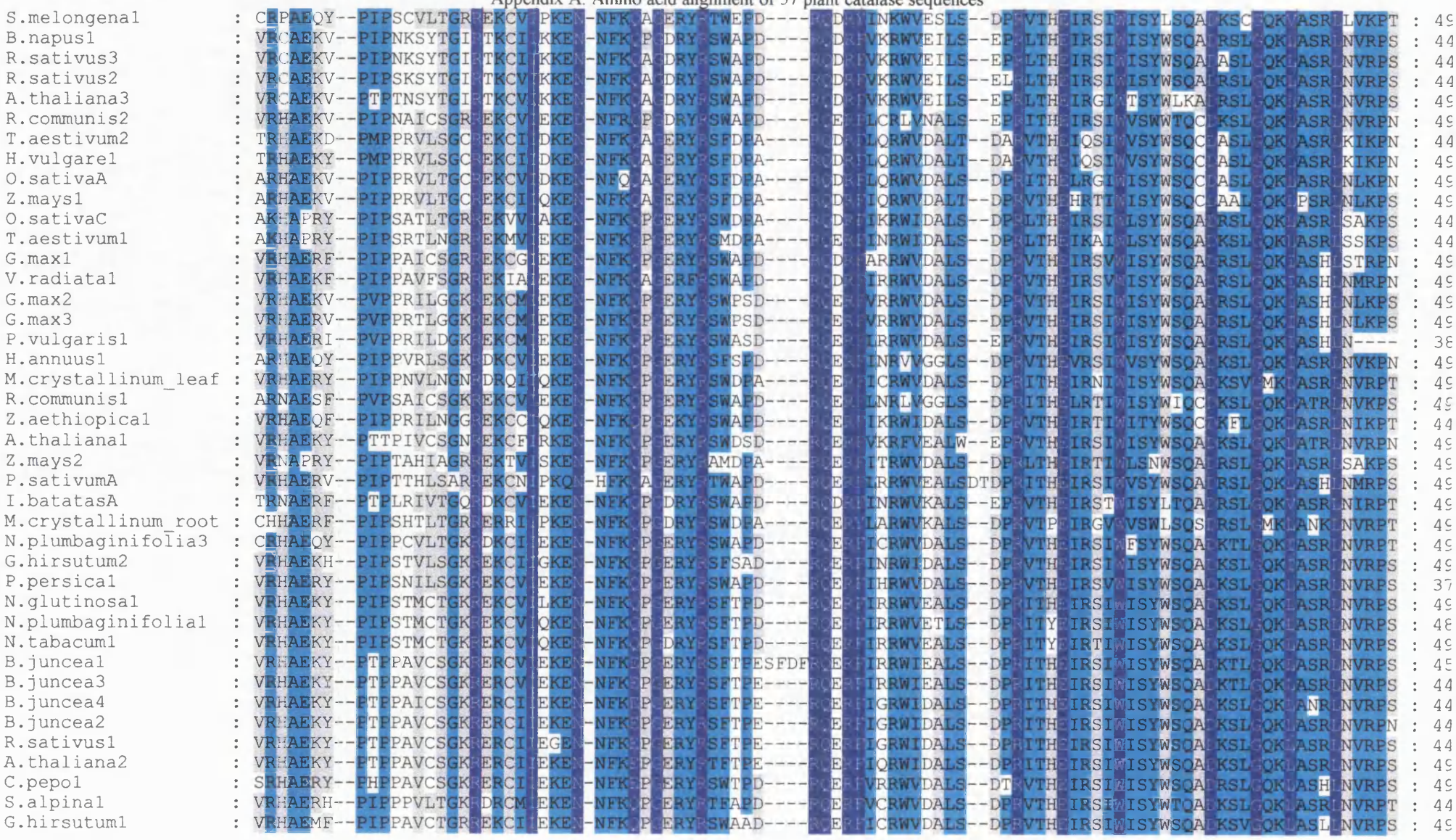
Appendix A. Amino acid alignment of 57 plant catalase sequences



Appendix A. Amino acid alignment of 57 plant catalase sequences



Appendix A. Amino acid alignment of 57 plant catalase sequences

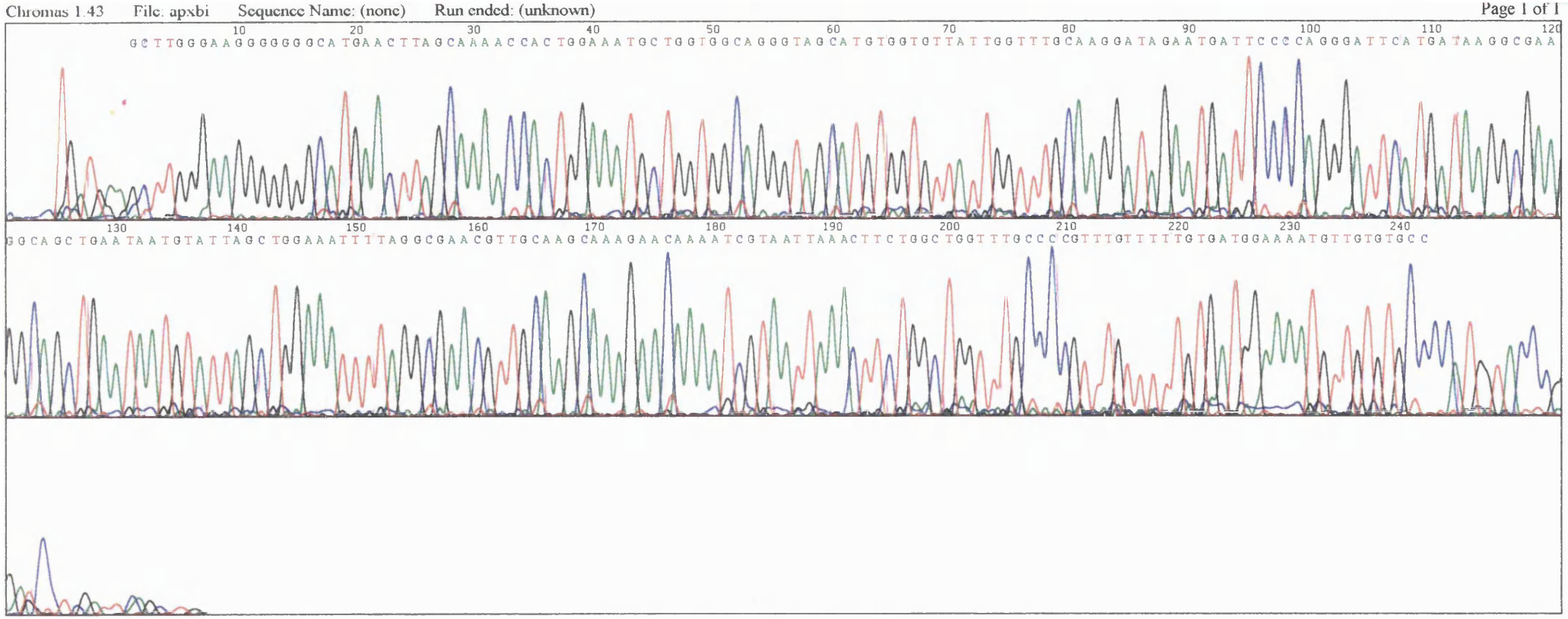


Appendix A. Amino acid alignment of 57 plant catalase sequences

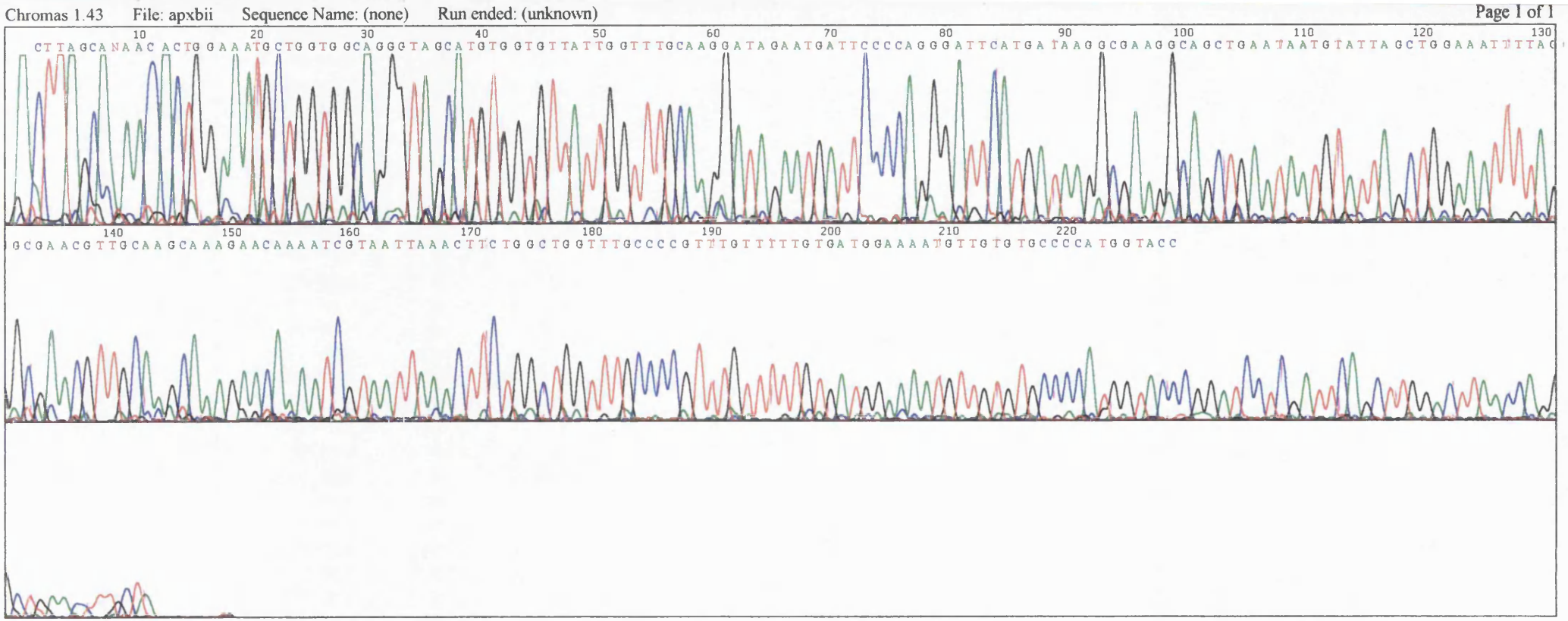
```
M.esculenta1          : I : 492
S.cereale1            : M : 448
H.vulgare2            : M : 494
O.sativa2             : M : 491
Z.mays3               : M : 495
C.reinharti2          : L : 448
C.reinharti1          : L : 449
C.pepo2               : M : 492
C.pepo3               : I : 492
N.tabacum2            : M : 492
N.sylvestris1         : M : 383
N.plumbaginifolia2    : M : 492
S.tuberosum2          : M : 447
L.esculentum1         : M : 492
S.tuberosum1          : M : 448
C.annuum1             : M : 448
S.melongena1          : M : 492
B.napus1              : I : 448
R.sativus3            : I : 448
R.sativus2            : I : 448
A.thaliana3           : I : 492
R.communis2           : I : 492
T.aestivum2           : M : 448
H.vulgare1            : M : 492
O.sativaA             : M : 492
Z.mays1               : M : 492
O.sativaC             : M : 448
T.aestivum1           : M : 448
G.max1                : I : 492
V.radiata1            : I : 492
G.max2                : I : 492
G.max3                : I : 492
P.vulgaris1           : - :   -
H.annuus1             : Y : 492
M.crystallinum_leaf   : M : 492
R.communis1           : I : 492
Z.aethiopica1         : L : 448
A.thaliana1           : F : 492
Z.mays2               : M : 491
P.sativumA            : I : 494
I.batatasA            : M : 492
M.crystallinum_root   : M : 493
```

Appendix A. Amino acid alignment of 57 plant catalase sequences

```
N.plumbaginifolia3  : M : 492
G.hirsutum2         : I : 492
P.persica1          : I : 379
N.glutinosa1        : I : 492
N.plumbaginifolia1  : I : 485
N.tabacum1          : I : 492
B.juncea1           : I : 452
B.juncea3           : I : 448
B.juncea4           : I : 448
B.juncea2           : I : 448
R.sativus1          : I : 447
A.thaliana2         : I : 492
C.pepo1             : I : 492
S.alpina1           : M : 448
G.hirsutum1         : I : 492
```

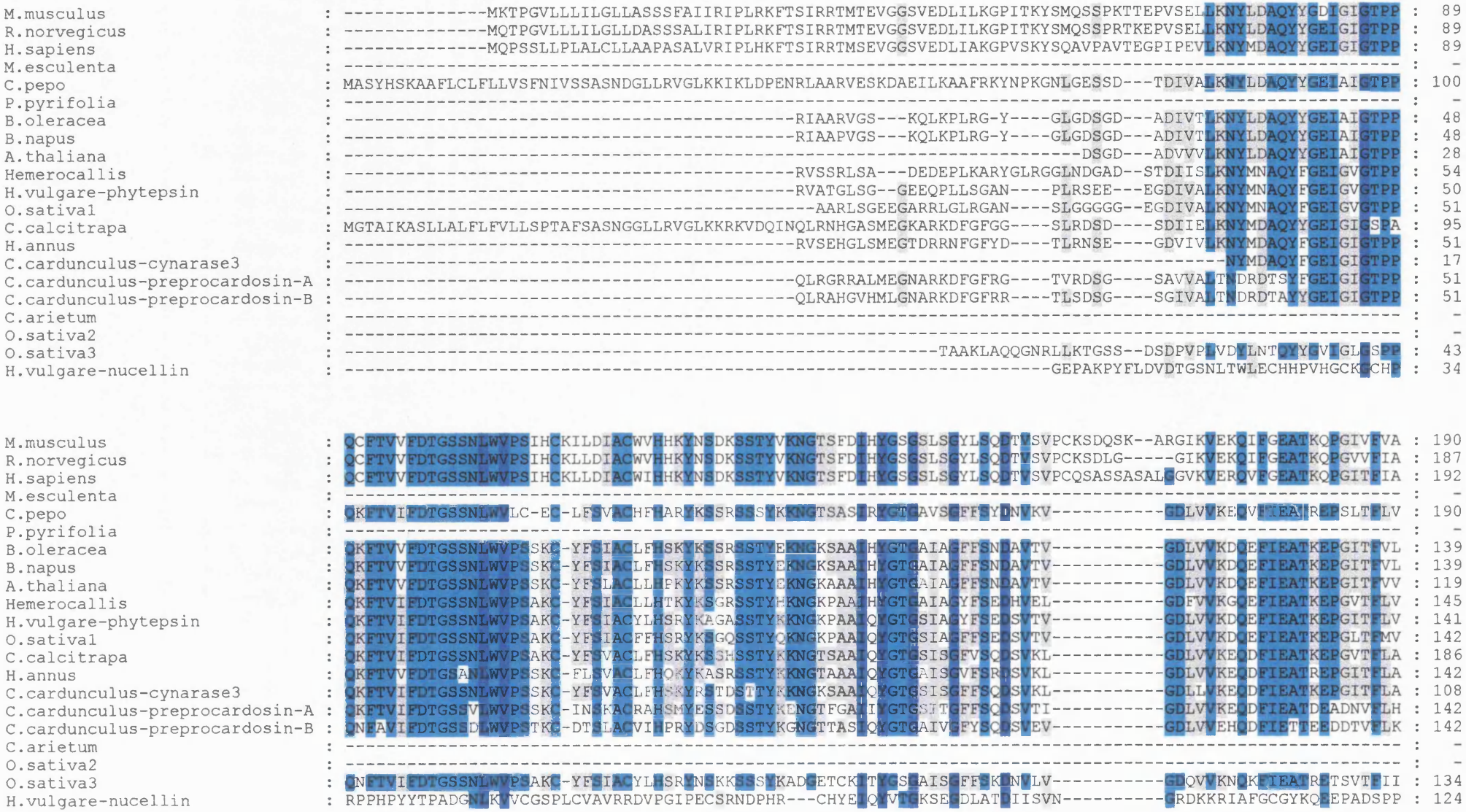


Appendix Bi: Sequencing of MecCuZnSOD-PCR. Sequence CuFF shown in reverse complement.

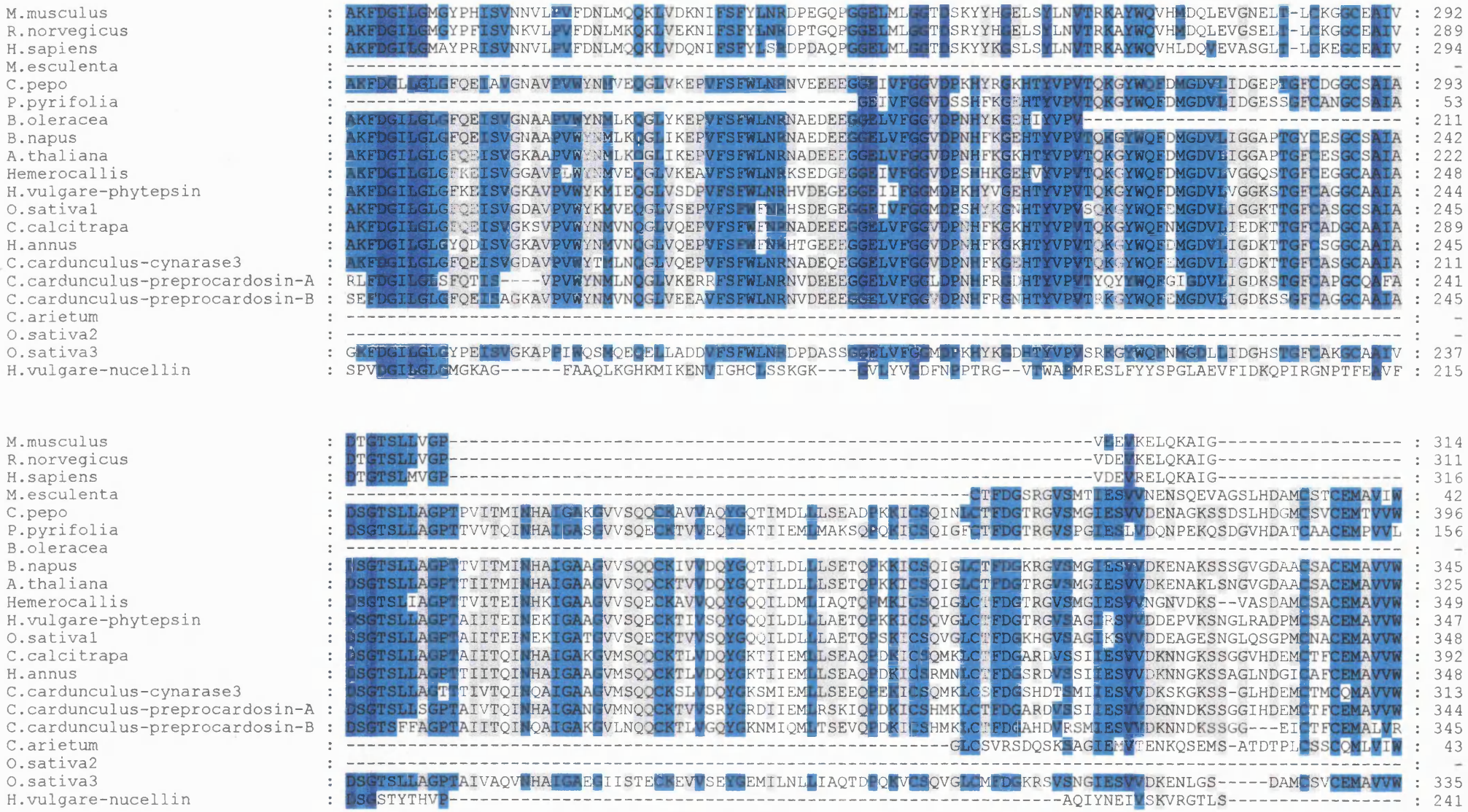


Appendix Bii: Sequencing of MecCuZnSOD-PCR.
Sequence of CuFR

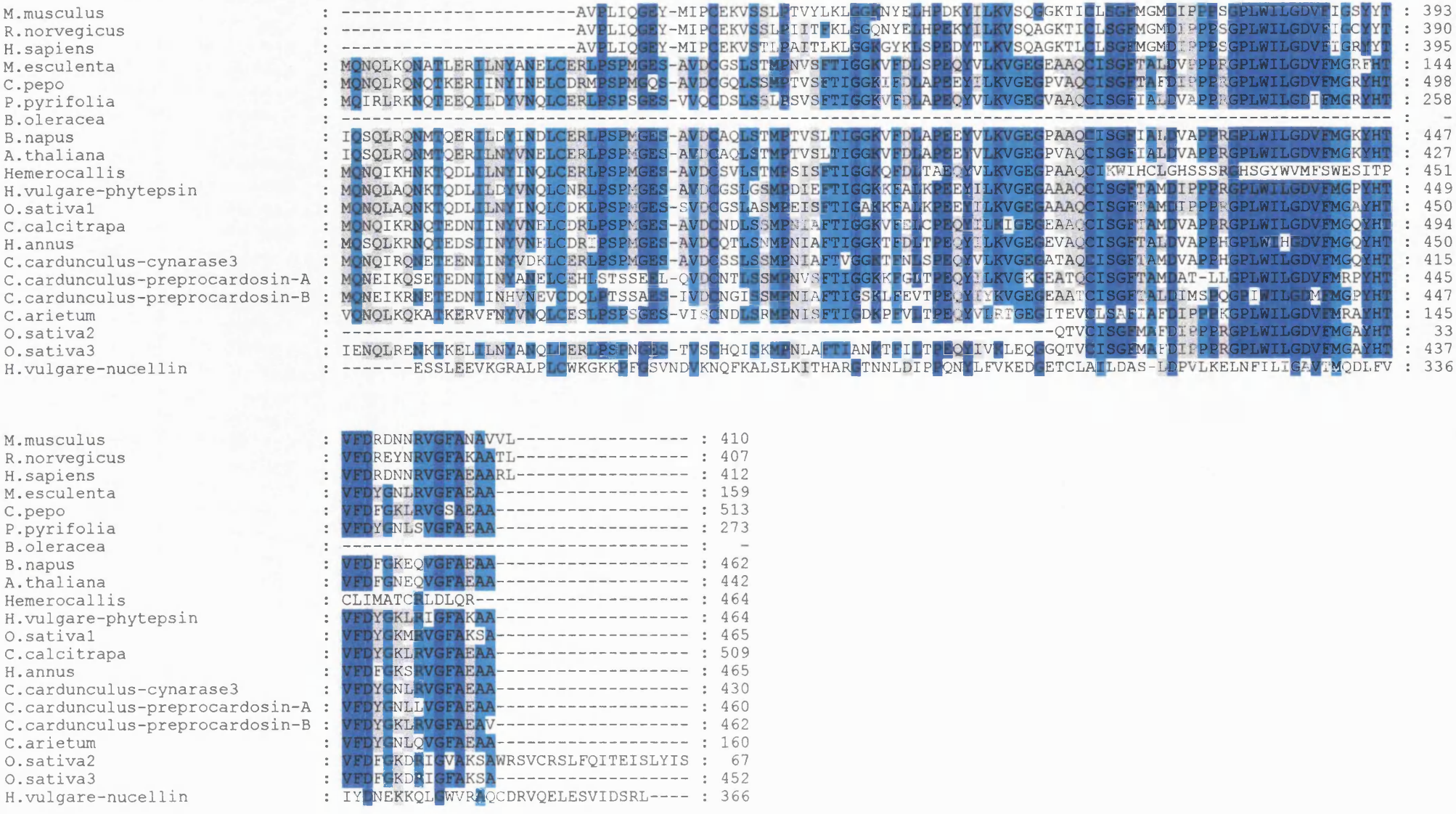
Appendix C. Amino acid alignment of plant and animal aspartic proteases

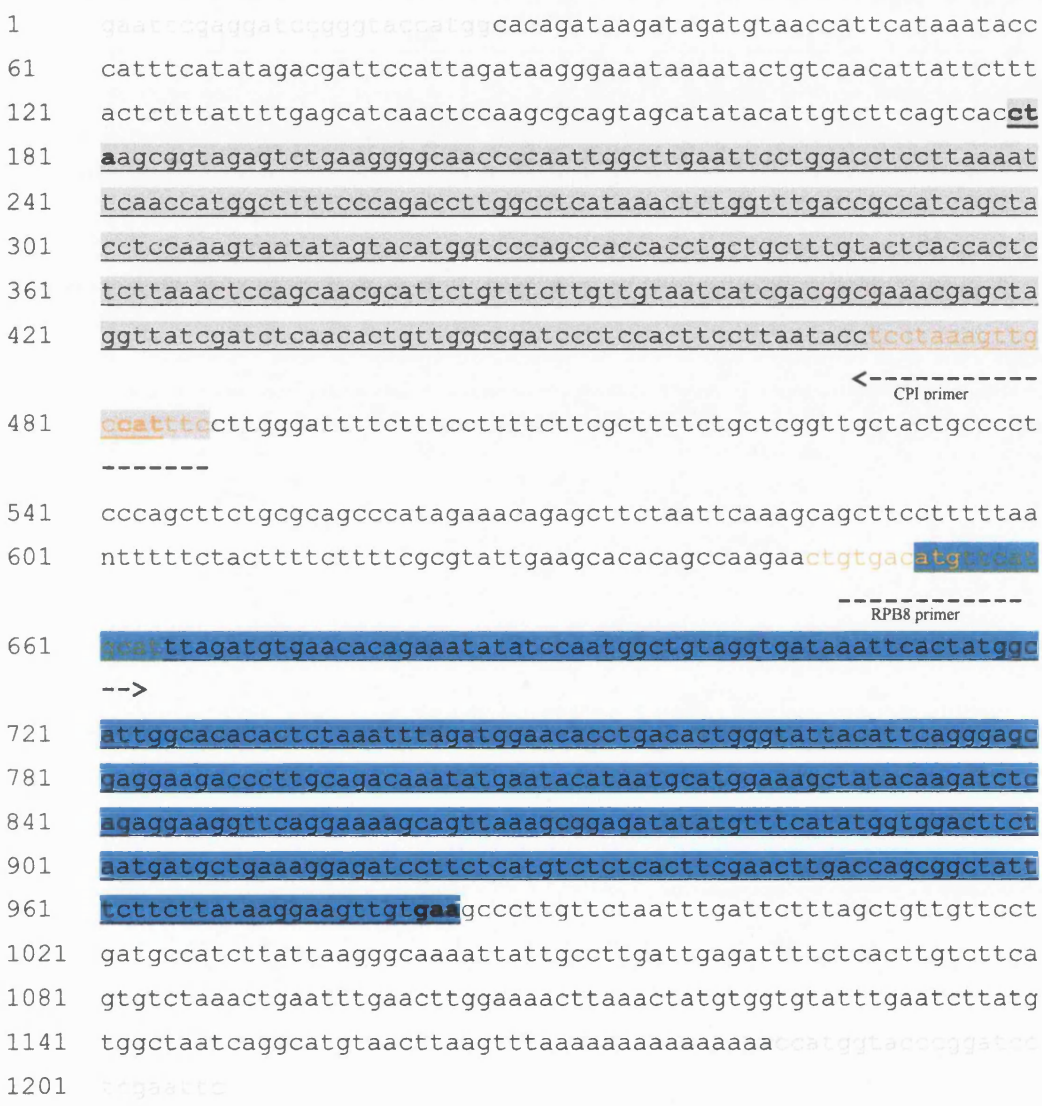


Appendix C. Amino acid alignment of plant and animal aspartic proteases



Appendix C. Amino acid alignment of plant and animal aspartic proteases





Appendix D Sequence of chimeric clone MecCPI/RPB8. The region encoding the cysteine protease inhibitor (cystatin) ORF reads 3'-5' and is highlighted in grey. The sequence encoding the RNA polymerase subunit RPB8 reads 5'-3' and is highlighted in pale blue. Start and stop codons of both ORFs are shown in bold. Sequences corresponding to primers used for PCR amplification are shown in orange text and indicated by arrows [CPI primer 5'-3' = GAAATGGCAACTTTAGGA, RPB8 primer 5'-3' = GTGACATGTTCATGCAT]. λ vector sequences are shown in grey text.